UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

Identification and comparison of *Culicoides* (Diptera: Ceratopogonidae), vectors and potential vectors of Bluetongue disease, captured near sylvatic animals and domestic cattle

Ana Maria Osório de Barros de Almeida Filipe

Mestrado em Biologia Humana e Ambiente

Dissertação orientada por:
Professora Doutora Maria Teresa Rebelo (FCUL)
Doutor David Wilson Russo Ramilo (FMV-ULisboa)

2020

ACKNOWLEDGEMENTS

I wish to express my most sincere gratitude to both my supervisors for giving me the opportunity to work under their supervision. To Prof. Dr. Maria Teresa Rebelo for being such a dedicated professor, answering all my questions very quickly and for helping me whenever I needed. Thank you for providing me with the tools that I needed for this project, for all the advices, critiques and valuable support.

To Dr. David Ramilo, without which I would not have come this far since he tirelessly helped me at any time, giving the necessary advice, wisdom and encouragement through this learning process. Thank you for your friendship, kindness, guidance, endless patience and, also the tireless corrections that have greatly improve this project. I could not ask for a better mentor.

I would like to extend my gratitude to all the colleagues from Faculty of Veterinary Medicine that, one way or another, helped on the development of this project. A special thanks to Dr. Sara Madeira, who kindly made the collection of *Culicoides* from Lisbon Zoo, an essential part of the work, and also for her enthusiasm and motivation. Thanks to Prof. Isabel Pereira da Fonseca and Dr. Elisabete Silva for advice and help in the identification of some malformations on *Culicoides*. To Dr. Lidia Gomes for her sympathy and kindness for sharing some laboratory supply and the greenhouse and to Dr. Marcos Santos for his constructive recommendations and for saving me in the lab so many times.

I am grateful to Prof. Francisco Pina Martins from Faculty of Sciences of the University of Lisbon, who was always present to assist me in last minute statistical doubts, specially questions related with *Rstudio* software.

I cannot forget all the precious support, help and fun times given from my colleagues from the Laboratory of Parasitology and Parasitic Diseases of the Faculty of Veterinary Medicine. So that, I would like to thank to Jorge Silva, Pedro Costa, Joana Sequeira, Sara Rocha e André Gomes. I was very lucky to be a part of such an intelligent, motivated and funny group of people. Also, I am grateful to Joana Matias for being always there, sharing her good mood, positivism, contagious energy and for sharing the same love for cats as I do.

I wish to express my gratitude to all my colleagues and friends from Faculty of Sciences of the University of Lisbon who have accompanied me during these years in Biology world. A special word of appreciation goes to a big friend of mine, André Costa, who inspires me every day regarding his conquests and for a better comprehension of the world and myself. Also, for always being there for me in the good and bad times during our academic life. Thank you for being who you are! Also, I would like to thank Marta Nascimento, Cátia Miranda, Ana Maria Lameiras, Joana Roque, Sofia Amorim, Luís Cavaco, Marta Macau and others for the moments of laugh and despair we shared.

Thanks to my second family, my closest friends from my hometown, Maria Beatriz Abalroado, Cristina Spinache, Alina Spinache, Catarina Catela, Flávia Courela, Sofia Patrício, Mariana Branco and Catarina Branco, who have always supported me, for their friendship, smiles, trust, knowledge, inspiration, motivation and source of strength in all the moments. I will never forget them. Also, I want to give a huge thank you to my friend, Margarida Falcato, although she is no longer with us, I know that she would be proud of my accomplishment.

I would like to thank my housemate, João Zacarias and my friend, João Costa for their company, support and for all the conversations (even though they did not realize anything I was saying…), but specially for being there for me, also as their cats, Pirata and Peludo.

Finally, I would like to show my appreciation to my family's unconditional support and love through all my life, with a special thank you for my mother Lidia, who shows me every day how strong a woman can be and for being always there for me. Also, I want to thank to my stepfather, António Costa, for being an extremely intelligent, inspiring person and for giving me the courage to fight every battle to achieve my professional and personal goals. To my grandmother, Maria Amélia Barros, for being the best person and for all the words of encouragement not only through my academic life, but also throughout my life, and for all the support, not only financial, but most importantly, emotional. To my eternal friend and grandfather, Gabriel Barros, who I know that would guide me throughout this journey and was always by my side. To my father, Carlos Filipe, for believing in me, his motivation and trust in me. To Zeca (my cat…) and Sebastião (my dog…), for their unconditional love and company, always making my weekends more fun. Lastly, I want to give a huge thank you for all the support during the years to my uncles Isabel Filipe, Chico Filipe and Francisco Filipe.

Thank you all for showing me that everything is possible, I am sure none of this would be possible without you. I am eternally grateful to you!

Identification and comparison of *Culicoides* (Diptera: Ceratopogonidae), vectors and potential vectors of Bluetongue disease, captured near sylvatic animals and domestic cattle

ABSTRACT

Culicoides biting midges (Diptera: Ceratopogonidae) have a major importance in animal and human health since they are vectors of several pathogens, like viruses (Bluetongue, African Horse Sickness and Schmallenberg), filarial nematodes (*Mansonella* spp.), among others.

Bluetongue is an arthropod-transmitted viral disease of domestic and sylvatic ruminants that was recognized and described more than 230 years ago in southern Africa. Bluetongue disease was enzootic throughout tropical and temperate regions of the world until 1998. Then, Bluetongue disease expanded to southern European countries and this spread has been driven by global warming, introduction of infected hosts or infected products from endemic regions that have allowed increased virus persistence, maintenance during the winter and the northward expansion of *Culicoides imicola* Kieffer, 1913, the main bluetongue virus vector. Female biting midges are responsible for huge economical losses worldwide and, since 2006, new Bluetongue-serotypes have also been reported from countries in Northern and Western Europe, where *C. imicola* is absent. However, little is known about *Culicoides* species associated with natural environments, and their role as vector species in wildlife should be investigated since opportunistic host feeding may facilitate virus transfer between wild and domestic hosts and even to humans.

The present work is based on *Culicoides* species captured near sylvatic animals (giraffes, zebras and birds) from Lisbon Zoo, between May 2018 and September 2019, and domestic cattle (cows) from Faculty of Veterinary Medicine, University of Lisbon captured between June 2019 and September 2019 (excluding August because the holiday period). Plus, older collections of cattle from a farm in Leiria District performed in 2010, during the National Entomologic Surveillance Program for Bluetongue disease in mainland Portugal (2005-2013), were used to compare those captured species with the most recent ones. *Culicoides* were collected with miniature CDC light traps. Then, biting midges were identified by morphological features, in order to understand the variation of *Culicoides* species in these collection sites.

Since knowledge of the blood-feeding behaviour of *Culicoides* midges is essential in assessing their vectorial competence and determining host preferences clarifies the roles of these species in the epidemiology of different diseases, the main aim of this work was to better understand how the distribution of *Culicoides* species is between sylvatic animals and domestic cattle. Specific goals include the comparison of *Culicoides* species captured near: i) sylvatic animals and domestic cattle; ii) three different sylvatic animals: giraffes, zebras and birds; iii) domestic cattle from: Leiria and Lisbon in two different time points and iv) detection and molecular identification of morphological anomalies in *Culicoides* from Obsoletus group.

The captures made between June and September 2019 (excluding August) were used to compare the captures made in Lisbon Zoo and in the Faculty of Veterinary Medicine, where from a total of 135 biting midges collected near sylvatic animals, eight *Culicoides* species were identified. On the other hand, from 158 *Culicoides* female specimens collected near domestic cattle, seven were identified. Also, this study showed the abundance of the main BTV vectors in Europe, *C. imicola* and Obsoletus group species, near sylvatic animals and domestic ruminants. That would support their putative role as bridge vectors for the transmission of the arboviruses between sylvatic animals and domestic cattle and once introduced, consequently, they will spread rapidly over large regions of Europe when appropriate environmental conditions and hosts are present. This is the first study in Portugal of *Culicoides* fauna captured near sylvatic animals and their comparison with those captured near domestic cattle.

The monitorization of *Culicoides* biting midges in zoo environment is important to avoid potential risk of outbreaks since imported animals could be reservoirs of diseases and zoo is a place where a large number of potential vectors may be present. In Lisbon Zoo, from a total of 1088 *Culicoides* biting midges, the biggest amount of *Culicoides* was collected near giraffes, representing 71% of all captures, followed by zebras with 28% and birds with 1%. *C. imicola* and species from Obsoletus group were the most captured ones.

When comparing the 9-year period in farm environments from Leiria in 2010 and Lisbon in 2019, it is important to notice that a drop in captured *Culicoides* species occurred. Probably this drop happened because of the hot and dry year, that may cause a deficit in the occurrence of favourable conditions for their emergence. However, Obsoletus group species represented in both years the most captured species, with 71% and 81% of all captures in 2010 and 2019, respectively. *C. punctatus* represented the second most captured species in both years, without significative differences in captures composition. Besides that, in both years, June was clearly the month with more captured specimens, following the characteristic pattern of seasonal dynamic of those species.

Since species inside Obsoletus group are very similar, their identification is not possible only by their wing pattern. So, in order to be identified they need to be dissected into different body parts. Through the detailed observation of these midges, it was possible to detect abnormal morphological structures. These abnormal structures may be due to the crossbreeding of close related species or the fact that they deposit 10 to 675 eggs, depending on species, that resulted in genetic errors that could cause morphological modifications. This project gives essential information concerning these aberrant characteristics, including a specimen with only one non-functional spermathecae in *C. obsoletus*, which was never reported before to the best of our knowledge. Furthermore, a molecular analysis of these specimens was made to understand their taxonomical position but, after several attempts, no results were obtained.

Known BTV vectors in Europe, as *C. imicola* and species from Obsoletus group (*C. obsoletus* (Meigen, 1818) and *C. scoticus* (Downes & Kettle, 1952), were the most captured species during this project. Their distribution and abundance are affected by abiotic factors, such as climate, temperature, wind exposure, soil type, surrounding vegetation and other factors, as the potential host proximity and exposure, density and size of hosts nearby the trap and the availability of larval habitats close to the traps. There is a big concern that climate change will lead to expansion of vector-borne diseases, as they are more sensitive to those factors, since these can affect tens of thousands of farms with high financial costs to farmers and countries and causing the death of millions of animals. Thus, such expansion may threaten human health and food security via effects on animal and crop health. Also, this project gives essential information concerning morphological modifications that can be observed in *Culicoides* biting midges, some of them referred for the first time.

Understanding the epidemiology of a disease allow us to take the correct measures to predict and prevent it, as well as enhancing our knowledge about the emergence of other vector-borne pathogens. A deeper knowledge of *Culicoides* fauna present in each region and their ecological preferences is required, so different control strategies can be applied efficiently.

Keywords: Bluetongue; *Culicoides*; domestic ruminants; morphology; sylvatic animals.

Identificação e comparação de espécies de *Culicoides* (Diptera: Ceratopogonidae), vetores e potenciais vetores da doença da Língua Azul, capturadas próximo de animais silváticos e bovinos domésticos

RESUMO ALARGADO

De acordo com a Organização Mundial de Saúde, as doenças transmitidas por vetores representam mais de 17% de todas as doenças infeciosas no mundo, originadas por agentes patogénicos como vírus, bactérias e parasitas. A distribuição destes agentes tem vindo a ser afetada pela recente combinação de fatores como a globalização comercial, o transporte rápido e acessível e o menor rigor das regulamentações internacionais de saúde em viagens, causando o aumento da disseminação de parasitas e de vetores para novas áreas geográficas, colocando populações de humanos e outros animais, anteriormente não expostos, em risco de infeção.

Os insetos do género *Culicoides* (Diptera: Ceratopogonidae) possuem uma grande importância na saúde animal e humana, uma vez que as fêmeas hematófagas são vetores de vários agentes patogénicos, como vírus (Língua Azul, Peste Equina Africana e Schmallenberg), filarídeos (*Mansonella* spp.), entre outros. Por sua vez, as fêmeas do género *Culicoides* são responsáveis por elevadas perdas económicas a nível mundial. Um conhecimento mais aprofundado da fauna de *Culicoides* presente em cada país é necessário, assim como as suas preferências ecológicas, de modo a que medidas possam ser aplicadas de modo mais eficaz e sejam criadas diferentes estratégias de controlo.

A doença da Língua Azul é uma doença viral, não contagiosa que afeta ruminantes domésticos e silváticos, que foi reconhecida e descrita há mais de 230 anos na África do Sul. O vírus da doença da Língua Azul era transmitido por vetores endémicos de regiões tropicais e temperadas até meados dos anos 90 do século XX. No entanto, nos últimos anos têm ocorrido mudanças drásticas na distribuição mundial destes vetores portadores de vírus, particularmente na Europa desde 1998, onde surgiu na região sul do continente, muito possivelmente devido à introdução de hospedeiros ou produtos infetados, provenientes de regiões endémicas, ou às alterações climáticas que permitiram a persistência do vírus durante o inverno e a expansão para norte de *Culicoides imicola* Kieffer, 1913, o principal vetor da doença. Além disso, desde 2006, novos casos da doença foram reportados em regiões mais a norte da Europa, onde a espécie *Culicoides imicola* não existe, indicando que outras espécies, como as pertencentes ao grupo Obsoletus, têm competência vetorial. No entanto, existem poucos estudos sobre a associação entre *Culicoides* e animais silváticos e, por isso, o papel destas espécies devia ser investigado, já que a alimentação oportunista em diferentes hospedeiros pode facilitar a transferência de vírus entre animais silváticos, domésticos e até em humanos.

Neste projeto, foram realizadas capturas de espécies perto de animais silváticos (girafas, zebras e aves) no Jardim Zoológico de Lisboa, entre maio de 2018 e setembro de 2019, e perto de bovinos domésticos da Faculdade de Medicina Veterinária da Universidade de Lisboa, entre junho e setembro de 2019 (excluindo o mês de agosto por ser período de férias de verão). Além dessas capturas, foram selecionadas capturas mais antigas de uma quinta em Leiria, realizadas em 2010 durante o Plano Entomológico de Vigilância Nacional para a doença da Língua Azul em Portugal (2005-2013), que foram utilizadas para comparar capturas mais antigas com as mais recentes.

Como o conhecimento dos padrões de alimentação das diferentes espécies de *Culicoides* é essencial para o conhecimento da sua capacidade vetorial e como a determinação das preferências de hospedeiros clarifica o papel destas espécies na epidemiologia das diferentes doenças, os objetivos específicos deste trabalho são compreender melhor a distribuição destas espécies de *Culicoides* entre: i) animais silváticos e bovinos domésticos; ii) três animais silváticos diferentes: girafas, zebras e aves; iii) bovinos domésticos: Leiria, capturas mais antigas e Lisboa, capturas mais recentes e iv) detetar e identificar através de análise molecular as anomalias morfológicas em *Culicoides* do grupo Obsoletus. Para tal, os

Culicoides foram capturados com recurso a armadilhas luminosas do tipo CDC, que são utilizadas durante o período noturno. Os insetos capturados foram analisados morfologicamente, mostrando a variação das espécies de *Culicoides* nestes locais.

As capturas realizadas nos meses entre junho, julho e setembro de 2019 no Jardim Zoológico de Lisboa e na Faculdade de Medicina Veterinária foram comparadas e, os resultados demonstraram que de um total de 135 exemplares capturados perto de animais silváticos, foram capturadas oito espécies diferentes. Por outro lado, foram identificadas, perto de bovinos domésticos, sete espécies de *Culicoides* de um total de 158 exemplares. Destas espécies que foram identificadas nos dois locais é de notar que a maior abundância vai para os principais vetores da doença na Europa, *C. imicola* e espécies pertencentes ao grupo Obsoletus (*C. obsoletus* e *C. scoticus*). Este resultado pode suportar o papel putativo destas espécies como ponte de transmissão de arbovírus entre animais silváticos e ruminantes domésticos que, consequentemente, uma vez introduzidos e sob as condições ideais, se disseminarão rapidamente por grandes regiões da Europa. Em Portugal, este é o primeiro estudo realizado da fauna de *Culicoides* capturados perto de animais silváticos e a sua comparação com capturas realizadas perto de ruminantes domésticos.

A monitorização da fauna de *Culicoides* em ambiente de zoo é importante para evitar o potencial risco de introdução de doenças vindas de animais importados. Neste contexto, as capturas foram realizadas no Jardim Zoológico de Lisboa entre maio de 2018 e setembro de 2019, de onde foram coletados 1088 exemplares, dos quais 71% capturados perto de girafas, 28% perto de zebras e apenas 1% perto de aves. Uma vez mais, as espécies mais capturadas foram *C. imicola* e aquelas pertencentes ao grupo Obsoletus.

Em relação à comparação dos resultados obtidos, com um distanciamento de nove anos nas explorações de gado de Leiria em 2010 e de Lisboa em 2019, é importante verificar que ocorreu uma queda acentuada no número de exemplares capturados. Esta redução, provavelmente, pode ter ocorrido devido ao ano quente e seco que se fez sentir em 2019 que dificultou a criação de condições favoráveis para o surgimento de *Culicoides*. Além disso, a composição de *Culicoides* nestas duas capturas revelou que as espécies pertencentes ao grupo Obsoletus foram as mais capturadas, representando 71% das capturas em 2010 e 81% das capturas em 2019. A segunda espécie mais capturada em ambos os anos foi *C. punctatus*, sem diferenças significativas na composição de ambas as capturas. Além disso, nestes dois anos, junho foi o mês com mais exemplares capturados, como esperado, tendo em conta os padrões da dinâmica sazonal destas espécies.

Tendo em conta que os indivíduos pertencentes ao grupo Obsoletus são muito semelhantes, a sua identificação apenas pelo padrão da asa não seria possível. Portanto, para separar estas espécies dentro do grupo Obsoletus é sempre necessário analisar as várias estruturas morfológicas e, por isso, foram detetadas alterações morfológicas. Estas alterações morfológicas podem dever-se ao cruzamento de espécies dentro deste grupo que são muito próximas e/ou ao facto de, dependendo da espécie, de depositarem entre 10 e 675 ovos, o que pode gerar a probabilidade de ocorrerem erros genéticos que podem causar estas malformações anatómicas. Neste projeto podem ser encontradas e descritas características morfológicas anormais, como é o caso de segmentos do palpo fundidos, exemplares com uma ou três espermatecas, em vez de duas funcionais e uma rudimentar. Com este trabalho é reportado pela primeira vez em Portugal um exemplar de *C. obsoletus* infértil que possuía apenas uma espermateca e esta não funcional. De forma a compreender a posição taxonómica destes espécimes com malformações anatómicas, foi realizada uma análise molecular, mas depois de várias tentativas não foram obtidos resultados.

No total das capturas realizadas neste projeto, as espécies mais prevalentes são as principais vetoras da doença da Língua Azul na Europa, *C. imicola* e espécies pertencentes ao grupo Obsoletus (*C. obsoletus* (Meigen, 1818) e *C. scoticus* (Downes & Kettle, 1952). A distribuição e abundância destas

espécies é afetada por fatores abióticos, como a temperatura, a exposição ao vento, entre outros fatores como a proximidade, exposição, densidade e tamanho de potenciais hospedeiros à armadilha, bem como a disponibilidade de habitats ideais ao crescimento das larvas de *Culicoides*.

Existe uma grande preocupação quanto às alterações climáticas e o potencial de permitirem a expansão de vários agentes patogénicos transmitidos por vetores, uma vez que estes são muito sensíveis ao clima. Os resultados deste projeto evidenciam e sustentam que, tendo em conta o impacto económico associado aos agentes patogénicos que transmitem e a constante expansão territorial de novos vetores, é importante realizar estudos entomológicos dos insetos do género *Culicoides*.

Concluindo, é importante ter conhecimento da epidemiologia de uma doença de forma a poder criar as medidas corretas de prevenção, e, além disso, o aumento do conhecimento nesta área permite a consciencialização do perigo deste tipo de doenças. Um conhecimento mais profundo na fauna de *Culicoides* presente em cada região e as suas preferências quanto a hospedeiros e ambiente é exigida para criar estratégias de controlo que sejam mais eficazmente aplicadas. Para este efeito, deveriam ser realizadas mais monitorizações ao longo do país e estudos onde a variação temporal das capturas, seja semanal ou ao longo de todo o ano, bem como a realização de estudos para se conseguir compreender melhor as anomalias morfológicas encontradas e a sua posição taxonómica, quando comparados com espécies já conhecidas.

Palavras-Chave: Animais silváticos; Bovinos domésticos; *Culicoides*; Língua Azul; Morfologia.

TABLE OF CONTENTS

FIGURE INDEX

TABLE INDEX

LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

A260	Absorbance at 260 nanometres
A280	Absorbance at 280 nanometres
AHSV	African Horse Sickness Virus
AinoV	Aino Virus
AKAV	Akabane Virus
bp	Base pair
BTD	Bluetongue Disease
BTV	Bluetongue Virus
BTV-1	Bluetongue virus serotype 1
BTV-2	Bluetongue virus serotype 2
BTV-4	Bluetongue virus serotype 4
BTV-8	Bluetongue virus serotype 8
C.	*Culicoides*
CA	California
CDC	Centers of Disease Control
CO_2	Carbon Dioxide
COI	Cytochrome Oxidase Subunit I
dH_2O	Distilled water
dNTPs	Deoxynucleoside triphosphates
DNA	Deoxyribonucleic Acid
Dr.	Doctor
EEV	Equine Encephalosis Virus
EHDV	Epizootic Haemorrhagic Disease Virus
e.g.	*exempli gratia*, for example
FMV-ULisbon	Faculty of Veterinary Medicine, University of Lisbon
g	Gram
H_0	Null Hypotesis
H_1	Alternative Hypotesis
H_2O	Water
IPMA	Instituto Português do Mar e da Atmosfera
LCS	Liquid Cristals
LZ	Lisbon Zoo
L/W	Length/Width
m	Meter
mA	Milliampere
$MgCl_2$	Magnesium Chloride
ml	Milliliter
mm	Millimeter
mM	Millimolar
mtDNA	Mitochondrial DNA
N	North; Size of statistical sample
ng	Nanogram
nm	Nanometres
MDV	Main Drain Virus
NESP	National Entomologic Surveillance Program
OIE	World Organization for Animal Health
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
rpm	Rotations per minute
SBV	Schmallenberg Virus
spp.	Species
TAE	Tris-acetate-Ethylenediaminetetraacetic acid
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
VBD	Vector-borne diseases
VBP	Vector-borne pathogens
vs.	*versus*, against
W	Watt; West
WHO	World Health Organization
µl	Microliter
µm	Micrometer
µM	Micromolar
USA	United States of America
º	Degree
ºC	Degree Celsius
'	Minute
"	Second
%	Per cent

1. INTRODUCTION

1.1. The importance of vector-borne diseases

Vector-borne diseases (VBD) represent more than 17% of all infectious diseases, caused by parasites, viruses and bacteria that are transmitted by vectors (e.g., mosquitoes, sandflies, triatomine bugs, blackflies, ticks, tsetse flies, mites, snails and lice) causing human and animal illnesses [1]. According to the World Health Organization (WHO) more than 700 000 human deaths globally are caused by VBD annually [1].

The distribution of VBD is determined by complex demographic, environmental and social factors. Among these factors, the recent globalization of trade allowed affordable and rapid transportation, and this factor combined with less control of international travel health regulations increased the spread of parasites and their vectors into previously unexposed geographical areas, which places humans and other animals at risk of infection [2].

Globally, vector-borne pathogens (VBP) account for a large proportion of emerging infectious diseases caused by anthropogenic (human-induced) changes that provides opportunities for vectors and parasites to expand their distribution in time or space or to evolve into more virulent or drug-resistant forms [2].

Among VBP, there are some kinds of pathogenic agents (e.g., Arboviruses) that need an arthropod vector to complete their life cycle and are transmitted to the vertebrate host during the arthropod blood-feeding process [3]. These arboviruses have significant importance in ruminants, deer and equines, and outbreaks are notifiable to the World Organization for Animal Health (OIE) [4].

There are several arthropods that can transmit different kinds of pathogenic agents, such as *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae) (Figure 1.1).

Figure 1.1: Illustration of a *Culicoides* spp. female specimen [5].

Culicoides are hematophagous pest and vectors of viruses, protozoans and filarial nematodes [6,7,8].

A major focus of research on *Culicoides* spp. is related to their roles as vectors of Bluetongue Virus (BTV), African Horse Sickness Virus (AHSV), Epizootic Haemorrhagic Disease Virus (EHDV) and Schmallenberg Virus (SBV), among others [6, 7]. Some species of genus *Culicoides* have been implicated in livestock diseases with economical, veterinary and public health importance [8].

Table 1.1. shows some viral diseases transmitted by *Culicoides* species of Western Europe.

Table 1.1: Viruses transmitted by *Culicoides* species present in Western Europe. Adapted from [9].

Virus	**Disease**	**Species Affected**	***Culicoides* species from Western Europe**	**References**
AHSV	African Horse Sickness	Horses, Mules, Donkeys and Zebras	*C. imicola* Kieffer, 1913 *C. obsoletus* (Meigen, 1818) *C. pulicaris* (Linnaeus, 1758)	[10]
AinoV	Aino Disease	Cattle and Sheep	*C. punctatus* (Meigen, 1804)	[11]
AKAV	Akabane Disease	Ruminants	*C. imicola* Kieffer, 1913 *C. nubeculosus* (Meigen, 1830)	[12]
BTV	Bluetongue Disease	Domestic and Sylvatic Ruminants	*C. achrayi* Kettle & Lawson, 1955 *C. chiopterus* (Meigen, 1830) *C. circumscriptus* Kieffer, 1918 *C. dewulfi* Goetghebuer, 1936 *C. imicola* Kieffer, 1913 *C. lupicaris* Downes & Kettle, 1952 *C. montanus* Shakirzjanova, 1962 *C. newsteadi* Austen, 1921 *C. obsoletus* (Meigen, 1818) *C. paolae* Boorman, 1996 *C. pulicaris* (Linnaeus, 1758) *C. punctatus* (Meigen, 1804) *C. scoticus* Downes & Kettle, 1952 Nubeculosus Complex	[13, 14]
EEV	Equine Encephalosis	All equine species	*C. imicola* Kieffer, 1913	[15]
EHDV	Epizootic Haemorrhagic Disease	Domestic and Sylvatic Ruminants	*C. circumscriptus* Kieffer, 1918 *C. festivipennis* Kieffer, 1914 *C. gejgelensis* Dzhafarov, 1914 *C. imicola* Kieffer, 1913 *C. kingi* Austen, 1912 *C. longipennis* Khalaf, 1957 *C. nubeculosus* (Meigen, 1830) *C. obsoletus* (Meigen, 1818) *C. pulicaris* (Linnaeus, 1758) *C. punctatus* (Meigen, 1804)	[16]
MDV	Main Drain Disease	Hares and Rabbits	*C. nubeculosus* (Meigen, 1830)	[17]
SBV	Schmallenberg disease	Ruminants	*C. chiopterus* (Meigen, 1830) *C. dewulfi* Goetghebuer, 1936 *C. obsoletus* (Meigen, 1818) *C. scoticus* Downes & Kettle, 1952	[18, 19]

1.2. *Culicoides* biting midges

1.2.1. *Culicoides* Taxonomy

According to Fauna Europaea website (2020) [20], the taxonomic position of *Culicoides* genus is as follows:

Kingdom	Animalia
Subkingdom	Eumetazoa
Phylum	Arthropoda
Subphylum	Hexapoda
Class	Insecta
Order	Diptera
Suborder	Nematocera
Infraorder	Culicomorpha
Superfamily	Chironomoidea
Family	Ceratopogonidae
Subfamily	Ceratopogoninae
Tribe	Culicoidini
Genus	*Culicoides*

Until 2016, a total of 1415 species were identified worldwide (1368 extant species and 47 fossil species) [21].

1.2.2. *Culicoides* Morphology

1.2.2.1. Immature *Culicoides*

The morphology of immature specimens has received less attention than that of adults, with only 13% of species being identifiable in their larval stages (e.g., *C. arakawai* (Arakawa, 1910), *C. bambusicola* Lutz, 1913 and *C. nubeculosus*) and 17% in their pupal stages (e.g., *C. parroti*, *C. festivipennis* and *C. obsoletus*), probably due to the relative difficulty in collecting immatures when compared to adults [22]. Immatures are mostly identified by rearing field-collected specimens to adult and the relative ease of rearing pupae compared to larvae has probably contributed to the higher number of species known as pupae than as larvae [23]. Commonly, breeding sites include intact dung pats (e.g., *C. chiopterus, C. dewulfi*), mud at the soil-water interface (e.g., *C. pulicaris*), and moist and highly organic soil substrates (e.g., old, composted manure mixed with soil) that do not usually have free-standing water (e.g., *C. imicola*, *C. obsoletus*) [24].

1.2.2.2. Adult *Culicoides*

Adult *Culicoides* are among the smallest blood-sucking flies, with body lengths that rarely exceed three mm [6]. In Figure 1.2 are described the body parts of a female *Culicoides imicola*.

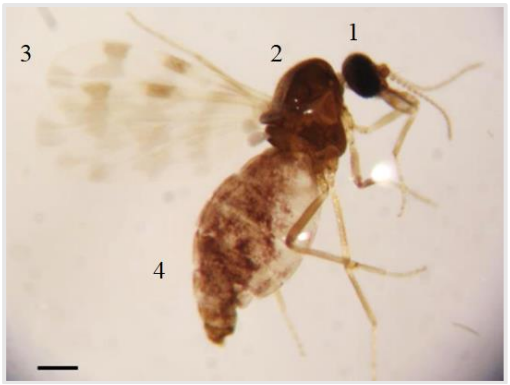


Figure 1.2: A female specimen of *Culicoides imicola*. Scale bar: 100 µm. Adapted from [9].
1-Head; 2- Thorax; 3- Wings; 4- Abdomen.

1.2.2.2.1. Head

The head of an adult *Culicoides* biting midge (Figure 1.3) is composed by the following anatomical structures: eyes, antennae, palpi, mouth parts, cibarium and posterior pharynx.

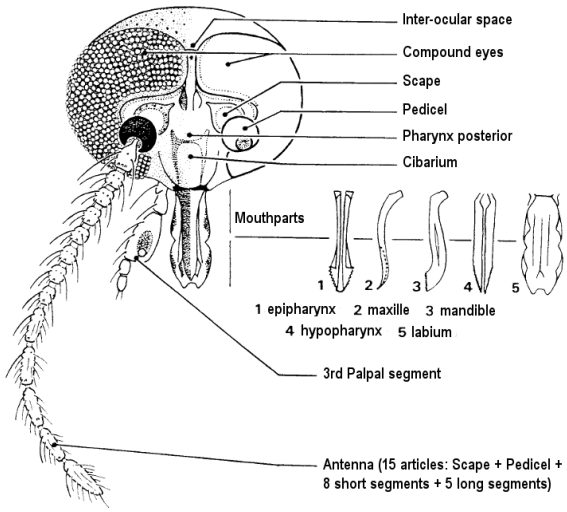


Figure 1.3: *Culicoides* spp. female's head [25].

The palpi or maxillary palps are structures arising from both sides of mouth parts. In both sexes, palpi are composed of five segments, being the first and the second fused. In order to identify *Culicoides* species, the 3rd palpus segment is sometimes used because of the size's variation from species to species and by the shape, number and depth of sensorial pits. This 3rd palpus segment has a sensorial organ named sensorial pit, which is composed by sensilla basiconica and these sensorial organs are specialized in detecting CO_2, feature that hematophagous females use to detect hosts [26].

Culicoides can also be identified by the type and number of antennal sensilla and by the presence or absence of interfacetal hairs [9, 27].

1.2.2.2.2. Thorax

Adult *Culicoides* thorax is composed of three segments: a small prothorax, a mesothorax, which subdivides in three parts: pre-scutum, scutum and scutellum, and a posterior portion, the metathorax [9].

The thorax comprises a pair of wings, a pair of halters and three pairs of legs, but the principal diagnostic feature used to distinguish *Culicoides* species are the wings. These have typical dark and light spots or patches, which localization is variable, although some species can have complete dark or light wings [9]. Figure 1.4 shows the cells and veins of a *Culicoides* biting midge wing.

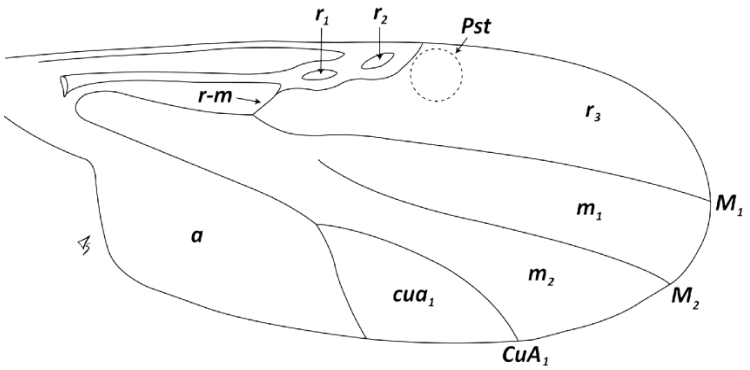


Figure 1.4: Cells and veins of a *Culicoides* biting midge wing (Diptera: Ceratopogonidae) [9].
r-m – Radio-medial crossvein; **r_1 –** First radial cell; **r_2 –** Second radial cell; **r_3 –** Third radial cell.
m_1 – First medial cell; **m_2 –** Second medial cell; **cua_1 –** Anterior cubital cell; **a –** Anal cell.
Pst – Poststigmatic pale spot; **M_1 –** First medial vein; **M_2** – Second medial vein.
CuA_1 – First branch of anterior cubital vein.

1.2.2.2.3. Abdomen

Abdomen of *Culicoides* midges is composed of 10 segments and the last segment has different conformation between females and males [26, 27] as shown in Figure 1.5.

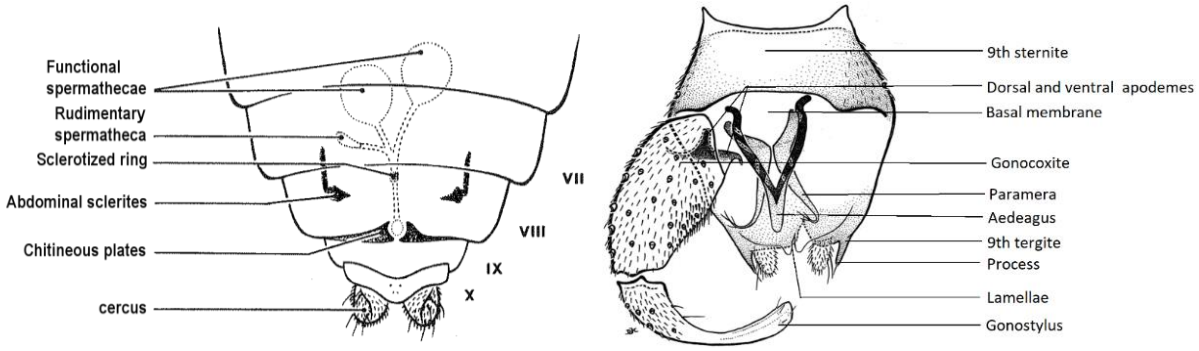


Figure 1.5: General view of the last abdominal segments of female (on the left) and male (on the right). Adapted from [28, 29].

Female *Culicoides* have one, two or three functional chitinous spermathecae (where sperm can be stored) in the abdomen, depending on species [9, 26, 30], and the shape and number of spermathecae can be used to identify *Culicoides* species [26].

1.2.3. *Culicoides* Anatomical Anomalies

Abnormal characteristics in *Culicoides* morphology are not commonly referred in scientific literature but they have an extremely importance since they are related to the quantity or to the aspect of structures with taxonomic importance and this may lead erroneous interpretations during classification [9]. Moreover, these anomalies in structures with specific functions can possibly affect insect's life activities [12].

Some studies have already been referred to *Culicoides* species from Central and South America, where abnormal structures are related to genetic or morphogenetic malformations, since they did not find neither parasitism or sexual anomalies. These abnormal characteristics included: absence of interocular hairs, fused flagellomeres, double sensorial pits in the 3rd palpus segment, fused palpus segments, deformed mandibular teeth, wing anomalies, two basal spines in 1st tarsomere, atrophied extra tarsus and three functional spermathecae in species where it was supposed to be only two functional spermathecae. Other studies from Nearctic and Paleartic ecozones [31, 32] reported three functional spermathecae in *C. achrayi* and *C. obsoletus* specimens, instead of those two typical functional spermathecae in these species [27].

1.2.4. *Culicoides* Biology

1.2.4.1. Life Cycle

The life cycle of *Culicoides* comprises the egg phase, four larval stages, a pupal form and the adult stage. The pupal form requires an amount of moisture-rich habitats for their development, such as free water or moisture and some species occur in both fresh water and estuarine environments. Breeding sites range from pools, streams, tree holes to saturated soil, animal dung and rotting vegetation [12].

Culicoides life cycle is described in Figure 1.6.

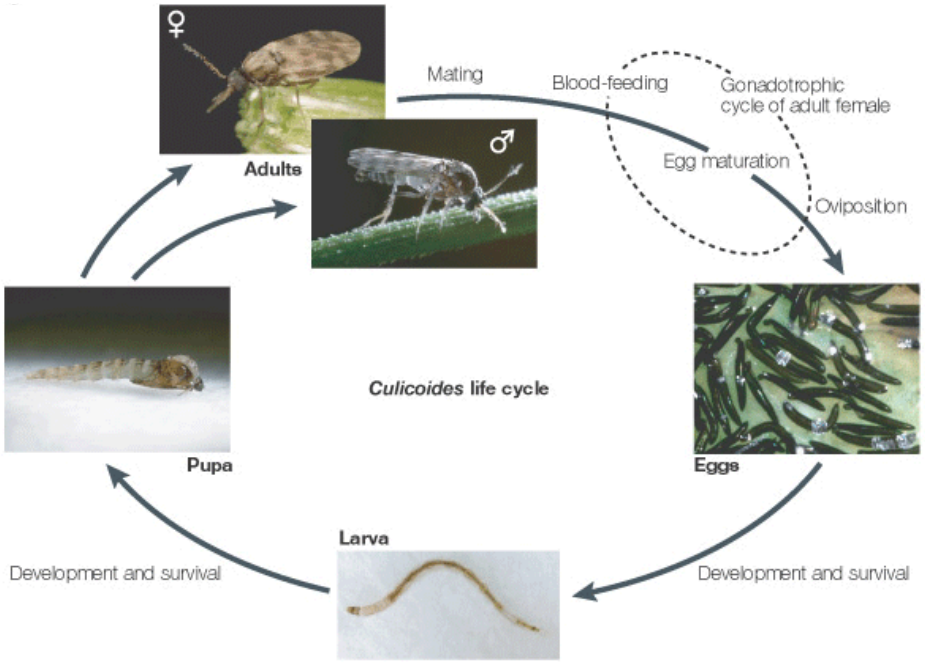


Figure 1.6: *Culicoides* life cycle [33].

Female biting midges require blood meals for the completion of the gonotrophic cycle. However, some species are autogenous and therefore may produce an initial batch of eggs without feeding, using reserves stored during the larval period [5]. Also, *Culicoides*' parity status provides information about their reproductive success, a relative estimation of female's age [34] and, when combined with the seasonal patterns of *Culicoides* abundance, it is an important key to assess the potential of pathogen transmission in an area [35]. Nulliparous females (which have not yet blood fed or laid eggs) have a lighter abdominal colour, while parous have completed at least one gonotrophic cycle (blood fed or laid eggs) and have a darker red pigment colour in abdomen. The *Culicoides imicola* female parity status is presented in Figure 1.7.

Figure 1.7: *Culicoides imicola* female parity status: top = unfed female, nulliparous; left = after blood-feeding; right = multiparous; bottom = with blood and eggs [9].

Although females depend on mammals or birds' blood for the maturation of their eggs, males do not feed on blood and can survive on nectar alone, being phytophagous [12].

1.2.4.2. Circadian rhythm and seasonality

The development of *Culicoides* is dependent of the temperature, resulting in a seasonal activity pattern in temperate regions that can variate from a few weeks to months. Biting midges can also overwinter during larval stages. In mild climatic zones, the insect numbers start to increase in late spring and early summer and usually peak in late summer or early autumn [6, 12].

With the arrival of lower temperatures, the number of active midges decreases dramatically. In general, adult biting midges are short-lived and only few individuals survive longer than 10 to 20 days and females may feed on multiple hosts. Usually, adult *Culicoides*' peak activity occurs around dawn and/or dusk [6].

1.2.4.3. Host preferences

Host preference is defined as an inherited tendency of an insect to blood-feed on vertebrate species (e.g.) and the knowledge of these preferences are pivotal. Since are important for the development of veterinary contingency plans to identify which species should be defined as vectors of a certain disease, to assess those transmission routes of VBD and prevent it to spread. *Culicoides* biting midges require an adaptation for different hosts since they are found in almost all parts of the world, living in a variety of habitats [36].

Previous studies on host feeding preferences have demonstrated that feeding rates have rarely been linked quantitatively to host availability and that a large proportion of *Culicoides* species are opportunistic in host selection [24]. The largest separation appears to be between avian, such as *C. circumscriptus* and *C. univittatus* Vimmer 1932, and mammalian feeders, such as *C. imicola*, *C. obsoletus, C. scoticus, C. pulicaris* and *C. punctatus*. These preferences could be explained by different breeding sites, whose coincide with bird or mammal habitats [9]. While a study suggested that *Culicoides* host preferences are based on variations in their sensorial morphology, such as the number and type of sensorial organs on the antenna and palps. These variations were the result of an adaptation associated with the relative size of large animals and consequently the amount of CO_2 produced compared to small birds [37]. Also, opportunistic host feeding may facilitate virus transfer between wild and domestic hosts or even to humans [24].

However, little is known about *Culicoides* species associated with natural environments, and their role as vector species in wildlife should be investigated [38].

1.2.5. *Culicoides* Distribution

Several factors influence the distribution of *Culicoides*, including both abiotic and biotic factors, such as climatic variables, primarily temperature and precipitation, followed by solar radiation, water vapor pressure, wind speed, land cover type and livestock distribution [39]. The major vector of the disease, *C. imicola* is a cosmopolitan biting midge species that has been reported from various geographic areas of the world, spanning its distribution from south Africa to southern Europe and from western Africa to southern China [39], and the recent northward expansion of *C. imicola* and unprecedent outbreaks of BTV and SBV viruses in southern Europe have been a major focus of research and surveillance.

The global distribution of *C. imicola* appears to be limited by climate, land cover and livestock distribution, as described in Figure 1.8.

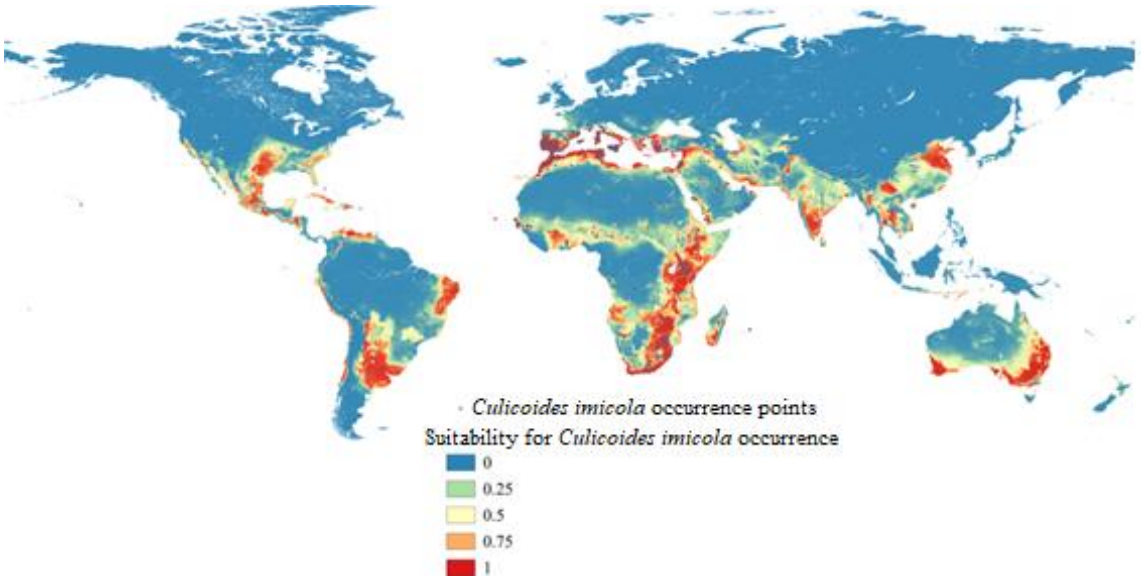


Figure 1.8: Actual estimated distribution of *C. imicola*. The scale indicates less suitable environments (cooler colours) and most suitable environments (warmer colours). Adapted from [39].

1.3. *Culicoides* and Bluetongue disease

1.3.1. Brief Historical Review of Bluetongue Disease

Bluetongue disease was first recognized and described in southern Africa by the French zoologist, Francois de Vaillant, between 1781 and 1784 [40]. Almost one hundred years later, in 1876, the disease was again reported in South Africa by Henning due to the introduction of various susceptible sheep breeds from Europe [41]. This disease was first referred to as fever, malarial catarrhal fever of sheep or epizootic malignant catarrhal fever of sheep [42].

Later, the first well recorded epidemic beyond the African continent dates back to 1943 in sheep from Cyprus, but there are indications that Bluetongue Disease (BTD) has been in this country since 1924 [43, 44]. Between 1943 and 1944 BTD was found in Israel [45] and, in 1948 it was reported in Texas, United States of America [46]. Between 1956 and 1957 a large epidemic broke out on the Iberian Peninsula, and subsequently BTD was also found in the Middle East, Asia and Southern European countries [43]. In Australia it first appeared in 1977 [47], and in South America it was found in the 1980s [48]. In 1998, the disease was recorded in Greece, in 1999 in Turkey and Bulgaria and, in 2000 it was found in Sardinia, Sicily, mainland Italy, Corsica, Menorca and Mallorca islands [49]. In 2001, BTD was first recorded in Croatia, Serbia, Montenegro, Kosovo and Macedonia, and the following year in Bosnia and Albania [49, 50]. Unexpectedly, in August 2006, BTD was also recorded in Central Europe (where *C. imicola* was absent) probably due multiple factors, such as introduction of infected hosts, infected products from endemic regions or global warming. This spread occurred first in The Netherlands and afterwards in Belgium and Germany also, between 2007 and 2008 in north of France and broke out to Northern European countries, such as United Kingdom, Denmark, Sweden and Norway [9, 51].

1.3.2. Bluetongue Disease

BTD is a viral, non-contagious disease, that affects domestic and wild ruminants (primarily sheep but also cattle, goats, buffalo, antelope, deer, elk and camels) that is transmitted by insects, particularly by biting midges of *Culicoides* genus [52]. The causing virus, BTV, is the type species of genus *Orbivirus*, *Reoviridae* family, and consists of a double-stranded RNA genome of 10 segments contained within three concentric shells of structural proteins that form the subcore, the outer core and the outer

capsid [53]. Nowadays, there are 28 different serotypes of BTV currently circulating and the ability of each strain to cause disease varies considerably [54].

The manifestations of BTD range from an unapparent to a fatal outcome depending on the serotype and strain of the virus and the species, breed and age of the infected animal (generally, older animals are more susceptible). The severity of disease varies among different species. The symptoms are most severe in sheep, resulting in deaths, weight loss and disruption in wool growth, with a morbidity that could be as high as 100% and mortality that averages from 2 to 30%, but which can be as high as 70%. The presentation and severity of clinical signs in cattle varies depending on the strain of virus but these animals often have a higher infection rate than sheep [55].

BTD occurs almost worldwide and is transmitted by approximately 30 species of biting midges of the genus *Culicoides*, such as *C. imicola*, *C. obsoletus*, *C. scoticus*, among others [56].

1.3.3. *Culicoides* as vectors of Bluetongue Disease

The terms "vectorial capacity" and "vector competence" are used interchangeably to describe the ability of a mosquito to act as disease vector. While vectorial capacity is influenced by variables as vector density and longevity, vector competence is a component of vectorial capacity, governed by intrinsic (genetic) factors that influence the ability of a vector to acquire, maintain and transmit a pathogen. Both environmental, behavioural, cellular and biochemical factors can play decisive roles in determining vectorial capacity and, any trait, such as host feeding preferences or susceptibility to pathogen infections will affect vector competence [57].

In BTV transmission between animals the insect vector is the key, since without the vector, the disease cannot spread from animal to animal. This process of transmission begins by sucking the blood of infected ruminants, where midges acquire BTV, which then replicates in their digestive tract. Virus progeny is then released into the haemocoel, where the secondary target organs (including the salivary glands) are infected. Virus replication occurs in the salivary glands and the transmission can take place. The whole cycle begins with the midge infection until transmission to a susceptible host (extrinsic incubation period) takes between 10 to 15 days at 25 ºC and individual vectors once infected usually remain so for life [6]. The process of BTV transmission cycle is described in Figure 1.9.

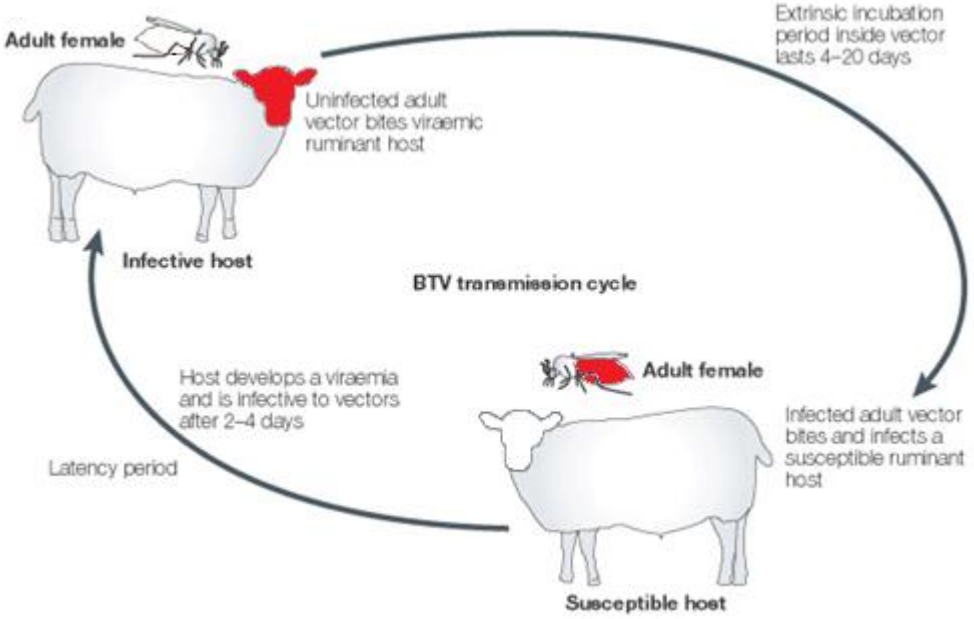


Figure 1.9: BTV transmission cycle. Adapted from [33].

The ability of biting midges to transmit BTV is markedly influenced by temperature, air humidity and total seasonal rainfall [6]. Inside the vectors, the virus can replicate with temperatures between 7 ºC and 28 ºC, with the intensity of replication growing with increasing temperatures [58].

Also, for several weeks, cattle may serve as a source of virus while displaying little or no clinical signs of disease and are often the preferred host for insect vectors. In previous studies, the virus has been found in semen from naturally infected bulls with BTV serotype 8 (BTV-8) and rams with BTV serotype 2 (BTV-2) and can be transmitted to susceptible cows and ewes, but this is not a significant mechanism of transmission [59, 60, 61]. The virus can also be transmitted to the foetus through the placenta (BTV-8), but it is not transferred through animal contact, milk consumption or wool [48, 62]

BTV presence in ruminant's blood is similar to the circulating half-life of ruminant erythrocytes, which is longer in cattle than in sheep [63].

The geographical distribution of the disease depends on the presence of certain species of *Culicoides*, e.g. *C. imicola, C. obsoletus, C. pulicaris, C. dewulfi, C. scoticus, C. punctatus* and *C. chiopterus*.

1.3.4. Bluetongue Disease in mainland Portugal

In Portugal, the first outbreak of BTV was reported in July 1956, caused by a strain of BTV-10, affecting mainly sheep flocks in the southern region of the country below Tagus River, excluding Algarve region, which was probably introduced by windborne *Culicoides* from Morocco [64, 65].

The Onderstepoort Veterinary Institute in South Africa supplied a monovalent live attenuated vaccine to use on sheep in the affected area of the country and the outbreak was largely eradicated two years later, although the region only declared free of disease in 1960 [66].

After a 44-year period of epizootic silence, BTV-4 was registered in national territory in areas of the central-west and southern regions of Portugal bordering Spain, in 2004, resulting in an outbreak. [65].

In 2005, the National Vigilance Entomologic Program of BTD in Portugal was created and the aim of this program was to understand the distribution of *Culicoides* species with vectorial competence in Portuguese territory, reporting to the competent authorities their presence, in order to prevent the dispersion of BTV in case of an outbreak [66].

Nowadays, serotypes BTV-1 and BTV-4 are present in mainland Portugal, with a major focus in Castelo Branco and Tagus river area, respectively, where vaccination is mandatory. However, voluntary vaccination is allowed of sheep and cattle present in the totality of mainland Portugal [67].

2. OBJECTIVES

Knowledge of the blood-feeding behavior of *Culicoides* midges is essential in assessing their vectorial competence and determining host preferences of *Culicoides* species clarifies the roles of these species in the epidemiology of different diseases. This understanding allows us to take the correct measures to predict and prevent such diseases, as well as enhancing our knowledge about the emergence of other vector-borne pathogens. A deeper knowledge of *Culicoides* fauna present in each region and their ecological preferences is required, so different control strategies can be applied efficiently.

The main aim of this work was to better understand the distribution of *Culicoides* species near sylvatic animals and domestic cattle since the knowledge of their ecological preferences is required to prevent outbreaks in Portugal. Specific goals included:

i) comparison of *Culicoides* species captured near sylvatic animals and domestic cattle (Lisbon);

ii) comparison of *Culicoides* biting midges collected near three different sylvatic animals (giraffes, zebras and birds);

iii) comparison of *Culicoides* species caught near domestic cattle (Leiria and Lisbon) in two different time points (2010 and 2019, respectively);

iv) detection and molecular analysis of morphological anomalies in *Culicoides* species from Obsoletus group.

3. MATERIAL AND METHODS

3.1. *Culicoides* Collection and Laboratorial Sampling

3.1.1. Sample Collection

Globally, to protect livestock from *Culicoides*, the most used method is the application of insecticides. Concerning their application during this project, it is known that in sylvatic animal's enclosures all rules of security hygiene and pest control are essential, meaning that spray insecticides were not regularly used, however could be used to control the population of cockroaches and ants, besides that, insect killer devices and adhesive screens were placed in some facilities to control insect flies. Also, animal's deworming was regularly made in sylvatic animals [68]. On the other hand, insecticides were not used in both collection sites where domestic cattle was present.

- ✓ **Sylvatic Animals**

One capture was made twice a month, between May 2018 and September 2019 at three different sites in the Lisbon Zoo (38°44'37.0"N 9°10'14.5"W) (LZ), near giraffes, zebras and birds (Figure 3.1) by the colleague Dr. Sara Madeira.

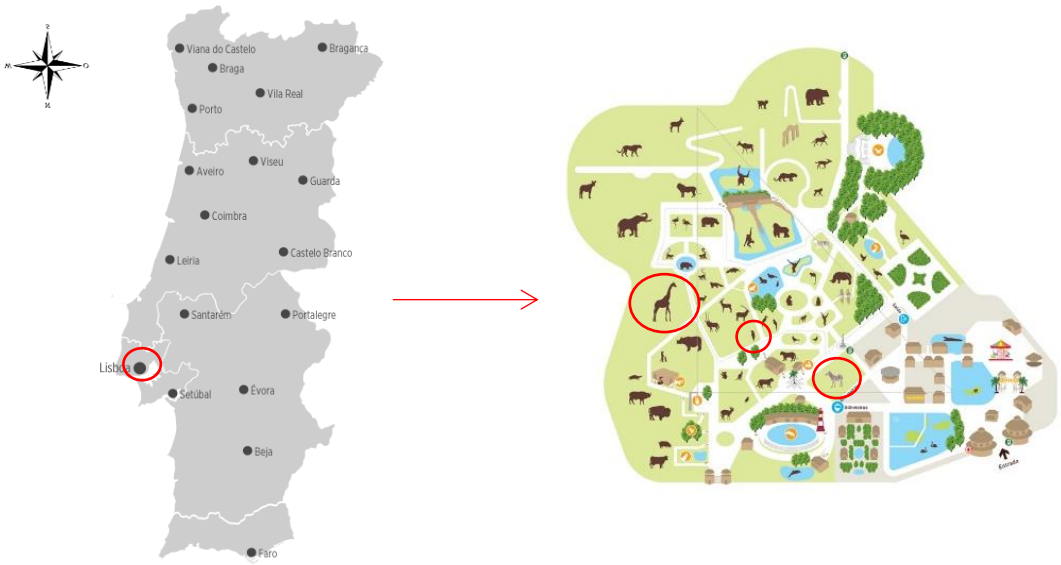


Figure 3.1: Map with the location of the traps near sylvatic animals (giraffes, zebras and birds) in LZ. Adapted from [69, 70].

The traps were placed in strategical spots, influenced by the conditions described at Table 3.1.

Table 3.1: Capture conditions near sylvatic animals in LZ, between May 2018 and September 2019.

Animal	Location of the trap	Water sources	Vegetation	Other animals	Human Presence	Cleaning
Giraffes	Tree Hole	Water fountain	Bushes and Trees	Yes, meerkats, muntjacs, hippopotamus, lemurs and rhinoceroses	Yes	Daily
Zebras	Under a Shed	Water and animal waste	Tall Trees	No	Yes	Daily
Birds	In a tree shadow with no wind, near parrots	Water fountain	Bushes and Trees	Yes, oryxes and impalas	Yes	Daily

✓ **Domestic Cattle**

For this study, two different insect collections were used: those that were captured in a farm from Leiria District (39°52'08.49"N 8°46'7.03"W) in 2010 during the National Entomologic Surveillance Program (NESP) for BTD and in a place near domestic cattle of Faculty of Veterinary Medicine, University of Lisbon, Lisbon (38º42'57.1"N 9º11'34.7"W) (FMV-ULisbon) in 2019 (Figure 3.2).

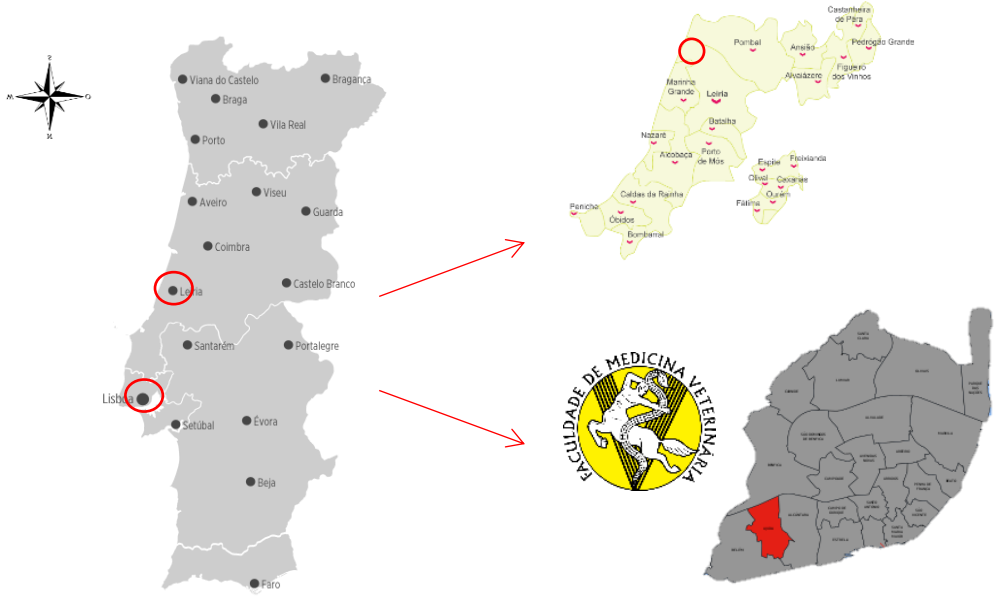


Figure 3.2: Map with the location of the traps near domestic cattle in a farm in Leiria (above) and in the Faculty of Veterinary Medicine in Lisbon (below). Adapted from [69, 71, 72].

The NESP for BTD was created in 2005, a year after the outbreak that occurred in mainland Portugal, to evaluate the distribution of different *Culicoides* species, so national authorities could take preventive measures in real time in case of another outbreak [9].

During NESP (2005-2013), several traps were placed in different cattle farms from Portugal. One of those farms was in Leiria District (39°52'08.49"N 8°46'7.03"W) and that place was selected for this study because, in those captures, the female midges had unique aberrant anatomical aspects [9].

The environmental conditions near the trap are described in Table 3.2.

Table 3.2: Capture conditions near domestic cattle in Leiria District in 2010.

Animal	Location of the trap	Water	Vegetation	Other animals	Human Presence	Cleaning
Cattle	Tree Hole	Water fountain and Atlantic Ocean 8 km away	Pine trees	No	Houses 2 km and Farms 6 km away	Sometimes, there was dry manure

A second place was selected to make captures near domestic cattle, in order to have recent samples of midges. These collections were compared with those from 2010 mentioned above, in order to understand the composition of *Culicoides* species in captures now and then.

Due to the localization of the domestic cattle enclosures (subject to disturbance by the public), captures were not made in the same place as those performed near sylvatic animals (LZ).

In FMV-ULisbon nine captures were made near cows, between June and September 2019, excepting for August because it is the month of summer break. The trap was placed in a strategical spot and the environmental conditions near it are described in Table 3.3.

Table 3.3: Capture conditions near domestic cattle in FMV-ULisbon between June and September 2019, excepting August.

Animal	Location of the trap	Water	Vegetation	Other animals	Human Presence	Cleaning
Cattle	Under a Shed	Drinking fountain	Variable	Yes, horses	Yes	Daily

3.1.1. Insect Sampling

Culicoides were collected using miniature CDC light traps (CDC miniature blacklight model 1212, John Hock, USA) fitted with 4 W UV bulbs, suction fans and LCS-2 Photoswitch Systems. CDC light traps were placed near the animals and 1,70 m above the ground, like demonstrated in Figure 3.3. This trap is capable of one-night collection [73].

All entities responsible for the trapping sites gave permission to place the traps.

Figure 3.3: Miniature CDC light trap being placed 1,70 m above the ground near domestic cattle in FMV-ULisbon.

In order to capture the insects, this trap automatically turns on the fan and the light every evening, at dusk, and turns off the light at dawn [73].

In both domestic cattle's captures, insects were collected into flasks containing ethanol 70% and a few drops of commercial detergent to fill 150 ml of final volume. A different process occurred in LZ, where the insects were collected into empty flasks by Dr. Sara Madeira, since in her project she needed to freeze culicids for morphological identification. Then, those flasks were brought to the Laboratory of Parasitology and Parasitic Diseases of the FMV-ULisbon to be identified.

3.2. Sample Selection and Identification

Concerning the domestic cattle's captures, when each flask was brought to the laboratory, the volume was poured and filtered to another flask with the aid of a gauze in order to count the captured insects. On the other hand, Dr. Sara Madeira's captures were delivered with separated and counted *Culicoides*, ready to be identified. The sample counting method in this laboratory is standardized and is described in Table 3.4.

Table 3.4: Sample counting method. Adapted from [7].

Number of insects	Dilution	Sample Count
< 2000	No	Total of insects
2000 - 5000	25%	$\frac{1}{4}$ of the insects
> 5000	2.5%	$\frac{1}{40}$ of the insects

All *Culicoides* specimens were stored in ethanol 70% before identification to species level.

3.2.1. Morphological Identification

- ✓ **Identification by the wing pattern**

The majority of *Culicoides* species can be identify by their wing pattern (light and dark areas) (Figure 3.4), and these structures were observed using stereomicroscope (Olympus SZ51).

An identification key with *Culicoides* species from Portugal was used [9].

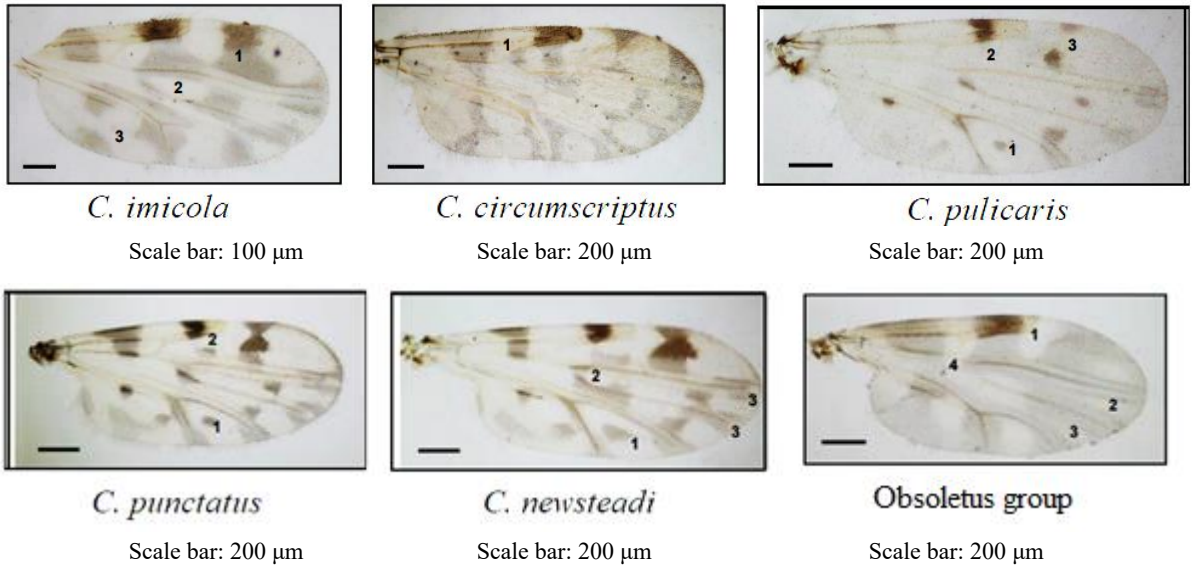


Figure 3.4: Different wing pattern observed in some *Culicoides* species. The numbers in the photos indicate wing spots useful for the identification of each species. Adapted from [9].

- ✓ **Identification of Females and Males by Abdominal Morphology**

The identification of male and female *Culicoides* specimens is done by observation of genital structures localized in the posterior part of the abdomen.

For this project, only the females were evaluated since males are phytophagous and not relevant for the transmission of the disease.

- ✓ **Preparation of *Culicoides* specimens from Obsoletus group**

When the identification of *Culicoides* was not possible only by the wing pattern (e.g., species from Obsoletus group), they were dissected into different body parts (head, one wing and abdomen) using 26 Gauge (0.404 mm diameter) needles, and then mounted in glass slides covered with a coverslip using Hoyer's medium, to clarify the internal structures, and dried in an incubator at 37 ºC for 3-4 days. The rest of the body (one wing, thorax and legs) was placed in an eppendorf with 96% ethanol for further molecular biology analysis.

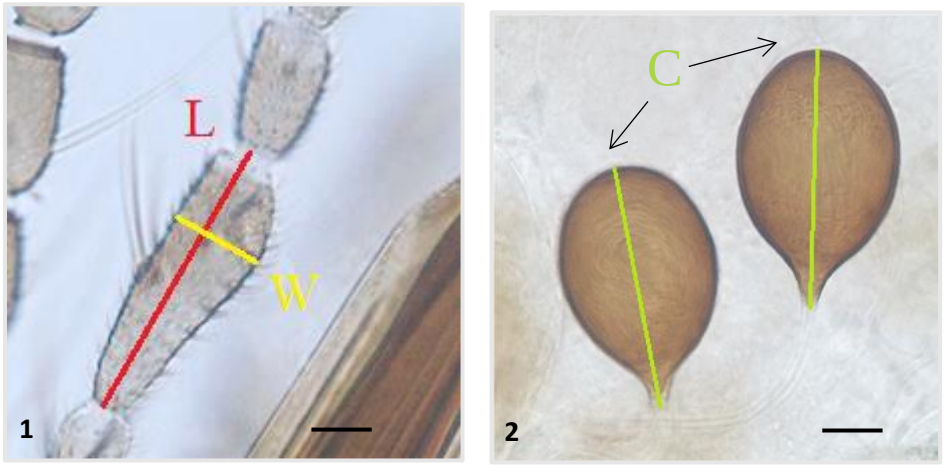
C. obsoletus, *C. scoticus* and *C. montanus* form the Obsoletus complex since females of these species are very difficult to distinguish between each other. However, for this project only *C. obsoletus* and *C. scoticus* were distinguished since the genetic distance between *C. obsoletus* and *C. montanus* was detected as the same order of magnitude as some intraspecific distances [74].

Some works refer that the format and the ratio length/width (L/W) of the 3rd palpus segment and spermathecae size are useful characteristics to distinguish Obsoletus group species (Table 3.5) [6, 13].

Table 3.5: Identification by ratio (Length/Width) of the 3rd palpus segment and by spermathecae size, based on [6].

***Culicoides* Species**	**L/W ratio**	**Spermathecae Size (µm)**
C. obsoletus	< 2.7	37.5 – 62.5
C. scoticus	> 2.7	57 – 95

These specimens were observed with an optical microscope, where different body structures can be clearly seen using an objective lens with 40x coupled with 10x eyepiece lens, resulting a total magnification of 400x (Olympus BX50 microscope). Photos were obtained with an Olympus DP10 camera (Figure 3.5).



Scale bar: 10 µm Scale bar: 17 µm

Figure 3.5: Measures of the internal structures that are useful for species identification (1- 3rd palpus segment: W- Palpus width and L- Palpus length; 2- spermathecae: C- spermathecae length). Total magnification of 400x.

After the measurements, glass slides were sealed with DPX Mountant and stored.

✓ **Preparation of *Culicoides* specimens from Nubeculosus group**

Nubeculosus group is composed of three species (*C. nubeculosus*, *C. puncticollis* (Becker, 1903) and *C. riethi* Kieffer, 1914) and, similarly to species from Obsoletus group, they cannot be distinguished by their wing pattern.

In order to identify midge's species, they were dissected into two body parts (one wing and abdomen) using 26 Gauge (0.404 mm diameter) needles, and then mounted in glass slides covered with a coverslip using Hoyer's medium, to clarify the internal structures, and dried in an incubator at 37 ºC for 3-4 days. The rest of the body (head, one wing, thorax and legs) was placed in an eppendorf with ethanol 96% for further molecular biology analysis.

The identification of these specimens was made by the size and shape of the spermathecae, as described in figure 3.6.

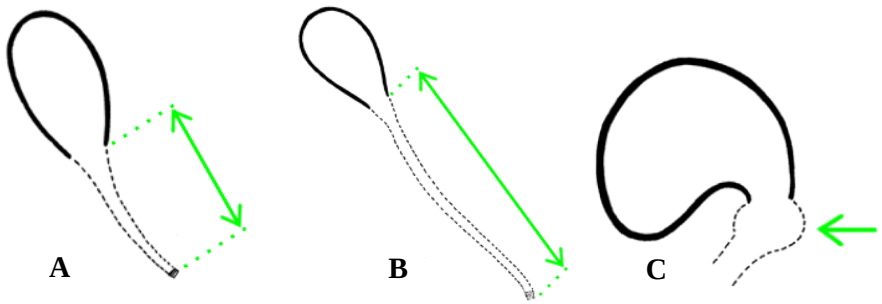


Figure 3.6: Illustration of spermathecae from: A) *Culicoides puncticollis*; B) *Culicoides riethi*.; C) *Culicoides nubeculosus*. Adapted from [24].

These body structures were observed with an optical microscope with a total magnification of 400x (Olympus BX50 microscope).

3.2.2. Molecular Identification of Obsoletus group species

Molecular biology is another useful tool to identify *Culicoides* species. This analysis was primarily used to understand the taxonomic position of female midges belonging to Obsoletus group with unique aberrant anatomical aspects and compare their genetic sequences with those of Obsoletus group species identified during this project.

- ✓ **DNA Extraction with Chelex**

Chelex 100 (Bio-Rad Laboratories, CA, USA) is a styrene-divinylbenzene copolymer containing paired iminodiacetate ions. It acts by chelating transition metal ions and the selectivity of this compound depends of the iminodiacetic acid. The Chelex resin became the method of choice for protocols requiring the rapid extraction of DNA from trace amounts of biological samples [75].

For each sample, a tube was used and filled with 100 µL of Chelex Solution (5g Chelex 100®/100 ml distilled water (dH_2O)). A *Culicoides* sample (composed of one wing, thorax and legs) was removed from their ethanol-filled eppendorf using a pipette and placed on a laboratory greenhouse for 5 minutes at 37 ºC. Then, using a needle (changed between samples to avoid DNA contamination), *Culicoides* body parts were put into the tube with Chelex solution and grinded. Then, 20 µl of Proteinase K was added to each solution. After that, a short centrifuge (spin-down) was made and the tubes were incubated for 1 hour at 56 ºC, then 30 minutes at 96 ºC. The next step was a centrifugation of the samples for 1 minute at 3200 rpm and, since Chelex beads inactivate *Taq* DNA polymerase, only supernatant was used for PCR analysis. Regarding DNA extraction, besides Chelex extraction, another two kits were tested, NZY Tissue Genomic DNA Isolation kit and ThermoFisher Scientific PureLink Genomic DNA Mini kit, to prevent the possible contamination of DNA by Chelex residuals. Furthermore, using a Nanodrop Spectrophotometer, DNA quantity and quality present in sample volumes of 1 µl of supernatant was measured. DNA concentration was estimated by measuring the absorbance at 260 nm (A_{260}) in order to understand if a dilution was necessary for the samples (maximum concentration of 50 ng/µl of DNA for PCR analysis). DNA purity was estimated using a A_{260}/A_{280} ratio, meaning that DNA was pure if the ratio value was between 1.7 and 2, and if necessary, a DNA purification was performed using a NZYGelpure kit. If the sample was not readily used, it was stored at -20 ºC.

- ✓ **Conventional Polymerase Chain Reaction (PCR)**

The polymerase chain reaction (PCR) was originally developed in 1983 by the American biochemist Kary Mullis, who received the Nobel Prize award in Chemistry in 1993 for his pioneering work [76]. Nowadays, PCR is used in Molecular Biology to make many copies (amplification) of small sections of DNA or a gene, allowing to generate thousands to millions of copies of a particular section of DNA from a very small amount of DNA.

For this project, a mitochondrial DNA (mtDNA) marker, Cytochrome Oxidase Subunit I (COI), was used for exhibiting a high level of interspecific polymorphism and few differences within species (intraspecific polymorphism) [29]. Thus, COI gene was amplified by PCR to obtain its genetic sequence.

The reagents used in the PCR mixture, as well as volumes, are shown in Table 3.6.

Table 3.6: Components of the reaction mixture (PCR).

	Reagents (Initial Concentration)	**Mix (Concentration)**	**1 Sample**
Master Mix	Pure H_2O		6 µl
	KAPA2G Robust HotStart ReadyMix 2X	1X	12.5 µl
	Primer C1-J-1718 (5 µM)	0.5 µM	2.5 µl
	Primer C1-N-2191 (5 µM)	0.5 µM	2.5 µl
	Sample, Negative or Positive Control	1 µl	1 µl
		Total	**25 µl**

Concerning the reagents, KAPA2G Robust HotStart ReadyMix KM5702 (KAPA Biossistems) contains an engineered *Taq* DNA polymerase enzyme, which aids in the addition of new bases to the newly formed sequences and has a uniquely formulated buffer to ensure the right conditions for the PCR reaction and DNA nucleotide bases, deoxynucleoside triphosphates (dNTPs), needed to construct the new DNA strand. It also contains 2 mM $MgCl_2$, important to increase *Taq* DNA polymerase activity by working as a cofactor and helping primers to bind at a specific location.

Primers, or short stretches of DNA initiate the PCR reaction, biding to both sides of the DNA section in order to be copied, were respectively [77]:

- ✓ Primer C1-J-1718 (forward): GGAGGATTTGGAAATTGATTAGT
- ✓ Primer C1-N-2191 (reverse): CAGGTAAAATTAAAATATAAACTTCTGG

The PCR analysis was based on a 522-bp (primers included) fragment of the gene COI by using these primers. Nucleotide 1 of the *Culicoides* sequence corresponds to COI nucleotide 250 and the last, nucleotide 472, corresponds to COI nucleotide 722 [26]. This analysis involves a process of heating and cooling thermal cycling (Table 3.7) which is carried out by *GeneAmp* PCR System 9700, Applied Biosystems, Foster City, CA thermocycler.

Table 3.7: PCR program conditions.

Program: PCR COI		
Temperature (ºC)	**Time (minutes)**	**Number of Cycles**
95	5	1
94[1] 54[2] 72[3]	1 1 1.5	35 or 40
72	7	1

[1] – Denaturing: when the double-stranded template DNA is heated to separate it into two single strands.

[2] – Annealing: when the temperature is lowered to enable the DNA primers to attach to the template DNA.

[3] – Extending: when the temperature is raised, and the new strand of DNA is made with the aid of the *Taq* DNA polymerase enzyme.

Moreover, for PCR, all the reagents were replaced to avoid contamination and a gradient of primers concentration was tested. Also, the thermocycler program was altered, reducing the number of cycles to 30 and a gradient of annealing temperatures was tested ([51.0-56.0] ºC).

- ✓ **Electrophoresis**

Typically, the electrophoresis is used to visualize DNA fragments, since it enables to distinguish DNA fragments of different lengths [78]. For this project, an agarose gel electrophoresis was made, where agarose powder was mixed with TAE buffer (Tris-acetate-Ethylenediaminetetraacetic acid) and heated to a high temperature until all of agarose powder had melted. The type of buffer used depends on the approximate size of the DNA fragments in the sample, since it conducts the electric current [79]. GelRed was added to the melted gel since it allows the fluoresce of strands for further exposition on UV light. Then, the melted gel was poured into a gel casting tray and a "comb" was placed at one end to make wells for the sample to be pipetted into. Once the gel had cooled and solidified (it will be opaque rather than transparent) the comb was removed. The gel was then placed into an electrophoresis tank and electrophoresis buffer was poured into the tank until the surface of the gel was covered, making sure that the orientation of the gel and positive and negative electrodes were correct. Afterwards, a DNA marker (Hyperladder IV) was generally loaded into the first well of the gel and, since fragments in the marker were of a known length, they can be used to help predict the approximate size of the fragments in the samples. Finally, a Loading buffer was added to the sample of DNA prior to electrophoresis to

increase the viscosity of the sample which will prevent it from floating out of the wells and so that the migration of the sample through the gel can be seen [79], and the prepared DNA samples were then pipetted in the remaining wells of the gel. For this project, two different agarose gels were used and TAE buffer was replaced too. The compounds of agarose gel can be seen on Table 3.8.

Table 3.8: Compounds of Agarose Gel.

Agarose Gel	
Concentration	1.5% (0.6g – 40 ml)
Electrophoresis Buffer	TAE 1x
PCR Products	8 µl (6 sample + 2 Loading Buffer)
GelRed	1.25 µl
Molecular weight marker: Hyperladder IV	5 µl

The electrical current was then turned on, following the conditions described in Table 3.9, so that the negatively charged DNA moves through the gel towards the positive side of the gel.

Table 3.9: Electrophoretic Conditions.

Electrophoresis Conditions	
Electrical Current	90 V
Amperage	400 mA
Time	60 minutes

After 60 minutes, the gel was placed in a UV-light box to visualize the fragments of DNA. Those fragments of DNA should have a defined length to be sent to STABvida (Monte da Caparica, Portugal) for DNA sequencing, in order to get the exact order of the four bases in a strand of DNA. The obtained COI sequences from specimens with anatomical malformations would be aligned, using *BioEdit* sequence alignment editor software (version 7.2), and compared with *C. obsoletus* and *C. scoticus* known sequences.

3.3. Statistical Analysis

Statistical Analysis was performed using *RStudio*® software (version 1.1.463).

Contingency Tables and Boxplots were created to see the frequency of the categorical variables and a Chi-Square test of independence was used to determine if there was a significant relationship between those two nominal (categorical) variables.

The Chi-Square test of independence is a non-parametric (distribution free) tool designed to analyze 2 groups of 2 different samples and allows the evaluation of both independent variables. Like all non-parametric statistics, it is robust with respect to the distribution of the data. It permits evaluation of both dichotomous independent variables [81].

Due to the small sample size ($N < 1000$) it is possible to use the Fisher's exact test of independence for some of the variables.

The null hypothesis (H_0) assumes that there are no association between the two variables and the alternative hypothesis (H_1) assumes that there are association between the two variables.

This test was used to compare the independence of:

Sylvatic animals vs. Domestic ruminants

Sylvatic animals: giraffes vs. zebras vs. birds

Domestic ruminants: Old captures vs. Recent captures

A *p*-value of < 0.05 was considered statistically significant in all the tests.

4. RESULTS

The data collected on this project and used for the following statistical analyses is located in annex 8.1, as well as the *Rstudio* script in Annex 8.2.

4.1. Sylvatic animals vs. Domestic ruminants

4.1.1. Distribution of *Culicoides* biting midges

A total of 293 *Culicoides* biting midges were collected in the period between June and September 2019, excepting August. From those sampling sites within the LZ and near cattle, captures resulted in 135 (mean: 16.88 per trap; median: 4 per trap) and 158 (mean: 22.57 per trap; median: 5 per trap) *Culicoides* female midges, respectively. The summary of the distribution of *Culicoides* species near these animals is represented in Figure 4.1 and detailed in Table 4.1.

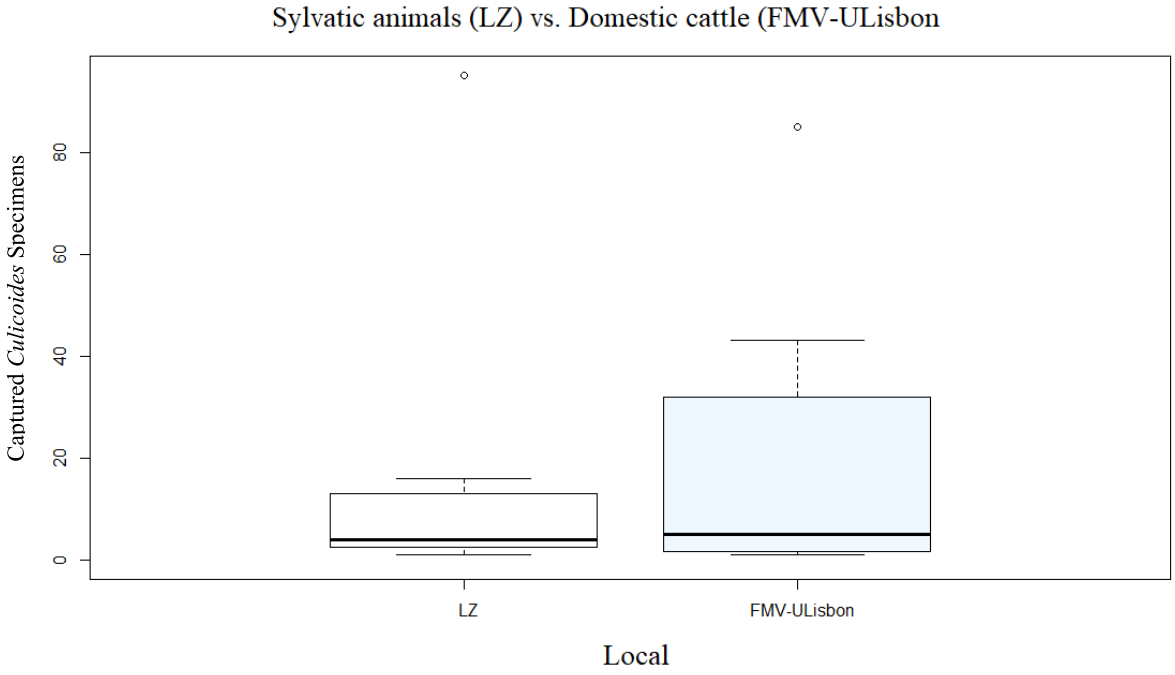


Figure 4.1: Boxplot representing the distribution of *Culicoides* specimens captured near sylvatic animals and domestic cattle between June and September 2019, excepting August.

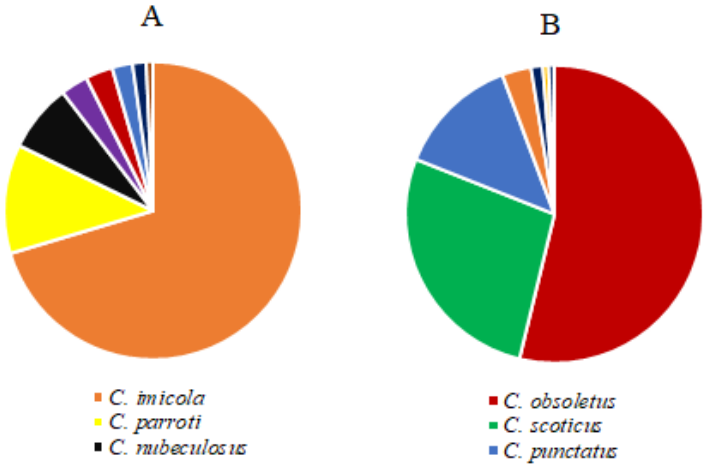
Table 4.1: Distribution of *Culicoides* specimens near sylvatic animals and domestic cattle in June, July and September.

	Minimum	Q1	Median	Q3	Maximum	Mean ($\bar{x}$)	Total (N)
Sylvatic animals	1	2.75	4	11.5	95	16.88	135
Domestic cattle	1	1.5	5	32	85	22.57	158

4.1.2. *Culicoides* species found near sylvatic animals and domestic cattle

The three most abundant *Culicoides* species caught near sylvatic animals and domestic cattle are represented in Figure 4.2 and the percentages were:

A) Sylvatic animals: *C. imicola* (70%), *C. parroti* Kieffer, 1992 (12%) and *C. nubeculosus* (7%).
B) Domestic cattle: *C. obsoletus* (54%), *C. scoticus* (27%) and *C. punctatus* (13%).



The other colours represent other *Culicoides* species captured near these animal groups.

Figure 4.2: Distribution of *Culicoides* species near: A) Sylvatic animals; B) Domestic cattle.

Eight *Culicoides* species were captured near sylvatic animals while seven species were captured near domestic ruminants during this time. The most marked differences were found in the proportions of the most abundant species, where the majority of *Culicoides* species caught are considered mammophilic. Plus, in wild animal's captures, *C. imicola*, the major vector of BTV, represented 70% and *C. obsoletus* represented 3% of all captures, while species from Obsoletus group, incriminated as vectors of BTV, were predominant near domestic cattle (81%), where *C. obsoletus* represented 54% and *C. imicola* represented only 3% of all captures.

4.1.3. Statistical Analysis: *Culicoides* species captured near sylvatic animals and domestic cattle

For these tests, the presence of different *Culicoides* species was associated with the capture site. Where the null hypothesis (H_0) assumes that there are no association between the capture site and the species captured near sylvatic animals and domestic cattle and the alternative hypothesis (H_1) assumes that there are association between the capture site and the species captured near sylvatic animals and domestic cattle.

From a total of 297 *Culicoides* biting midges, the H_0 was:

- Chi-square test (*p*-value): 1.031e-46 (Rejected).
- Fisher's exact test (*p*-value): 0.0004998 (Rejected).

The overall statistical analysis showed that there is association between the distribution of *Culicoides* species and the local where they were captured (which had a *p*-value below a pre-defined threshold of $p < 0.05$).

4.2. Sylvatic Animals: birds vs. giraffes vs. zebras

4.2.1. Distribution of *Culicoides* biting midges

A total of 69 captures and 1088 *Culicoides* biting midges were collected in LZ between May 2018 and September 2019 at three different sites, 20 captures near birds, 27 captures near giraffes and 25 captures near zebras, resulting in 14 (mean: 2.33 per trap; median: 1 per trap), 767 (mean: 47.94 per trap; median: 6.5 per trap) and 307 (mean: 76.75 per trap; median: 30.5 per trap) *Culicoides* female midges, respectively. The summary of the distribution of *Culicoides* species captured near these animals is represented in Figure 4.3 and detailed in Table 4.2.

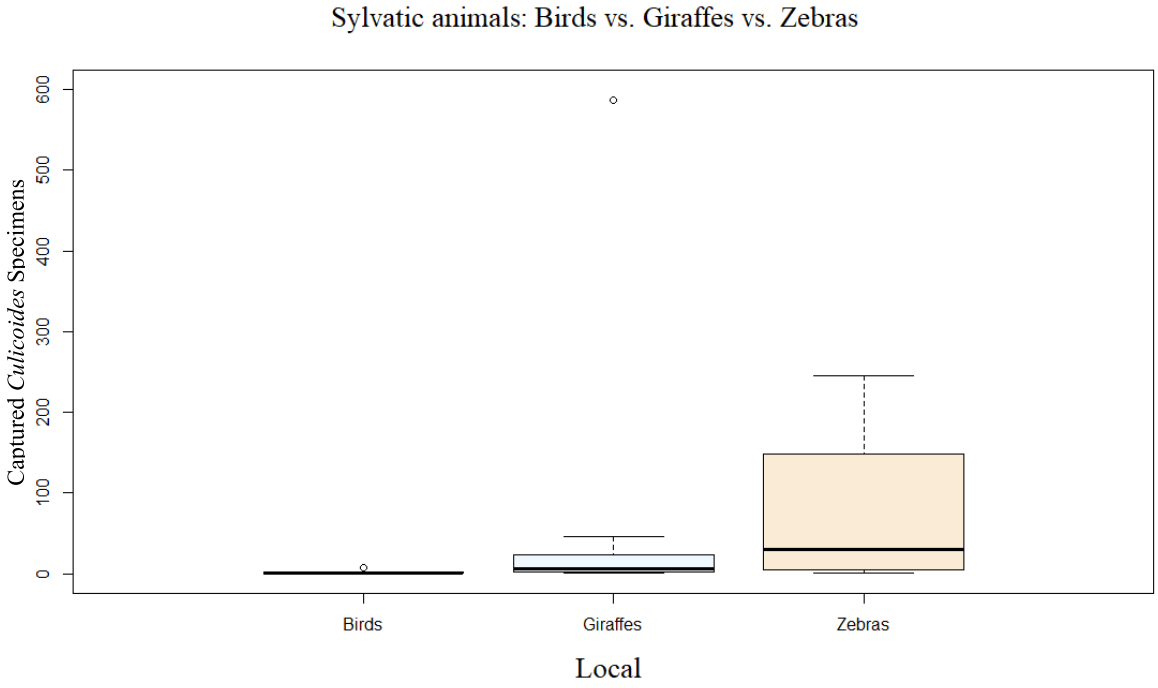


Figure 4.3: Boxplot representing the distribution of *Culicoides* biting midges near Sylvatic animals: birds, giraffes and zebras, between May 2018 and September 2019.

Table 4.2: Distribution of *Culicoides* species captured near Sylvatic Animals: birds, giraffes and zebras, between May 2018 and September 2019.

	Minimum	**Q1**	**Median**	**Q3**	**Maximum**	**Mean ($\bar{x}$)**	**Total (N)**
Birds	1	1	1	1.75	8	2.33	14
Giraffes	1	2.75	6.5	22.5	587	47.94	767
Zebras	1	7	30.5	100.25	245	76.75	307

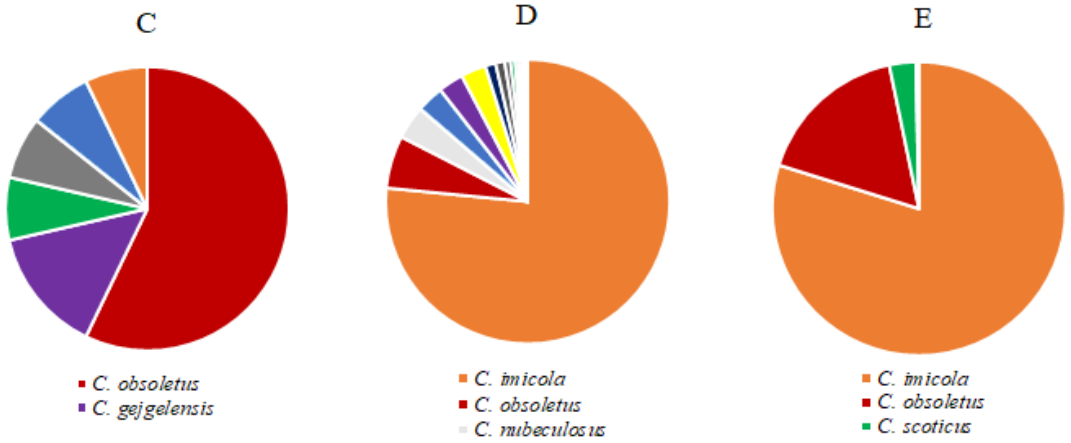
4.2.2. *Culicoides* species found near birds, giraffes and zebras

The most common *Culicoides* species captured near different sylvatic animals are represented in Figure 4.4 and the percentages were:

C) Birds: *C. obsoletus* (57%) and *C. gejgelensis* (15%).

D) Giraffes: *C. imicola* (77%), *C. obsoletus* (6%) and *C. nubeculosus* (4%).

E) Zebras: *C. imicola* (80%), *C. obsoletus* (17%) and *C. scoticus* (3%).



The other colours represent other *Culicoides* species captured near these animal groups.

Figure 4.4: Distribution of *Culicoides* species near: C) Birds; D) Giraffes; E) Zebras.

A total of six different species were captured near birds, sixteen near giraffes and five near zebras. From these three sylvatic animals the biggest amount of *Culicoides* was collected near giraffes, representing 71% of all captures, followed by zebras with 28% and birds with 1%.

4.2.3. Statistical Analysis: *Culicoides* species captured near sylvatic animals

For these tests, the presence of different *Culicoides* species was associated with the capture site. Where the null hypothesis (H_0) assumes that there are no association between the capture site and the species captured near those three sylvatic animals (birds, giraffes and zebras) and the alternative hypothesis (H_1) assumes that there are association between the capture site and the species captured near those three sylvatic animals (birds, giraffes and zebras).

From a total of 1088 *Culicoides* biting midges, the H_0 was:

- Chi-square test (*p*-value): 4.582e-18 (Rejected).
- Fisher's exact test (*p*-value): 0.0004998 (Rejected).

The overall statistical analysis showed that there is association between the distribution of *Culicoides* species and the local where they were captured (which had a *p*-value below a pre-defined threshold of $p < 0.05$).

4.3. Domestic Ruminants: Leiria vs. Lisbon (FMV-ULisbon)

4.3.1. Distribution of *Culicoides* biting midges

A total of 3505 *Culicoides* biting midges were collected near domestic cattle and identified. From those, 3347 were captured during NESP (2005-2013) in Leiria District (mean: 418.4 per trap; median: 29.5 per trap) between June and September 2010, excluding August, and 158 were captured in FMV-ULisbon (mean: 26.33 per trap; median: 3.5 per trap) in the same period, but 9 years later.

In Figure 4.5 is represented the summary of the distribution of *Culicoides* species captured near these animals and detailed in Table 4.3.

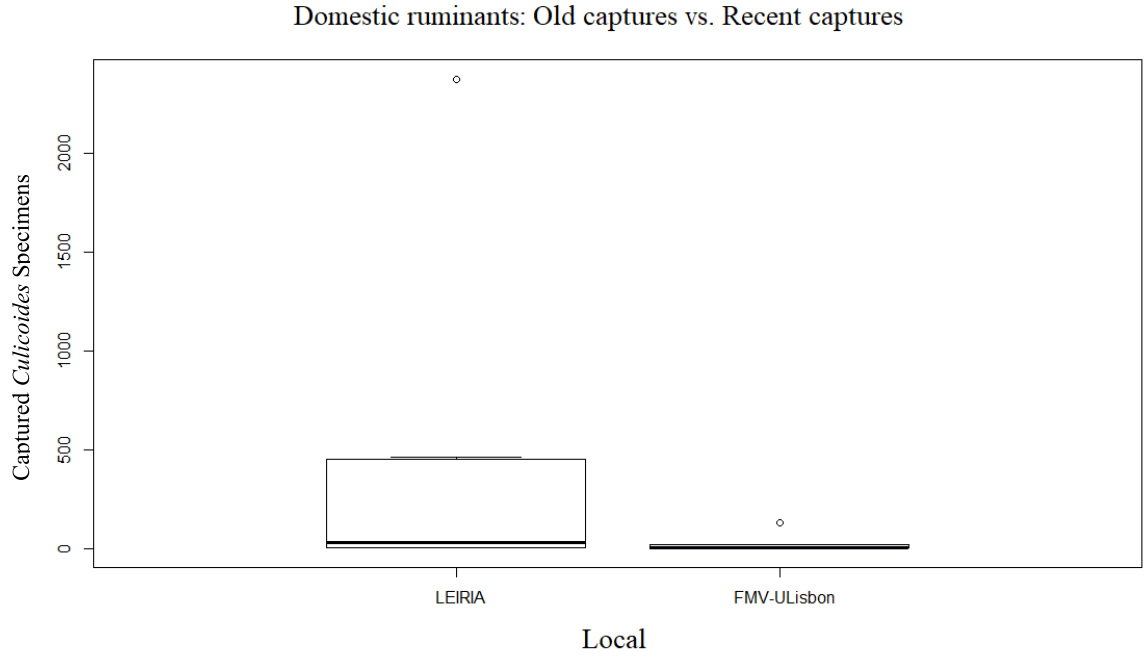


Figure 4.5: Boxplot representing the distribution of *Culicoides* biting midges in Leiria and FMV-ULisbon performed between June and September 2010 and 2019 (excluding August), respectively.

Table 4.3: Distribution of *Culicoides* specimens, between June and September (excluding August), near domestic cattle: old captures (2010) vs. recent captures (2019).

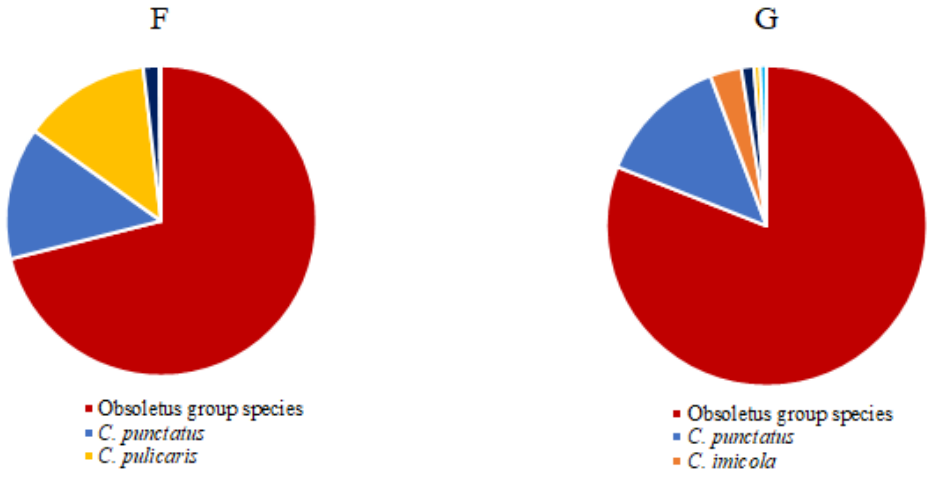
	Minimum	Q1	Median	Q3	Maximum	Mean ($\bar{x}$)	Total (N)
Leiria	1	1	29.5	449.2	2378	418.4	3347
FMV-ULisbon	1	1.25	3.5	17	128	26.33	158

4.3.2. *Culicoides* species found near domestic cattle from Leiria and Lisbon (FMV-ULisbon)

The most common *Culicoides* species captured near domestic cattle are represented in Figure 4.6 and the percentages are:

F) Leiria: Obsoletus group species (71%), *C. punctatus* (14%) and *C. pulicaris* (13%).

G) FMV-ULisbon: Obsoletus group species (81%), *C. punctatus* (13%) and *C. imicola* (3%).



The other colours represent other *Culicoides* species captured in these locations.

Figure 4.6: Distribution of *Culicoides* Specimens in: F) Leiria; G) FMV-ULisbon; Captured between June and September 2010 and 2019 (excluding August), respectively.

Besides Obsoletus group, a total of seven different species were captured in Leiria and five in FMV-ULisbon and the species composition varied significantly among years.

Species from Obsoletus group are the most abundant and widespread species in both captures, increasing from 71% to 81% in composition within a period of 9 years. However, without significative differences in both captures composition, *C. punctatus* is the second most caught species.

Understanding variations in space and time of vector species populations and their host-feeding pattern is important since it can contribute to a better knowledge of their respective roles in pathogen transmission, and, consequently, design the accurate vector control strategies or measures. This variation is showed in Figure 4.7.

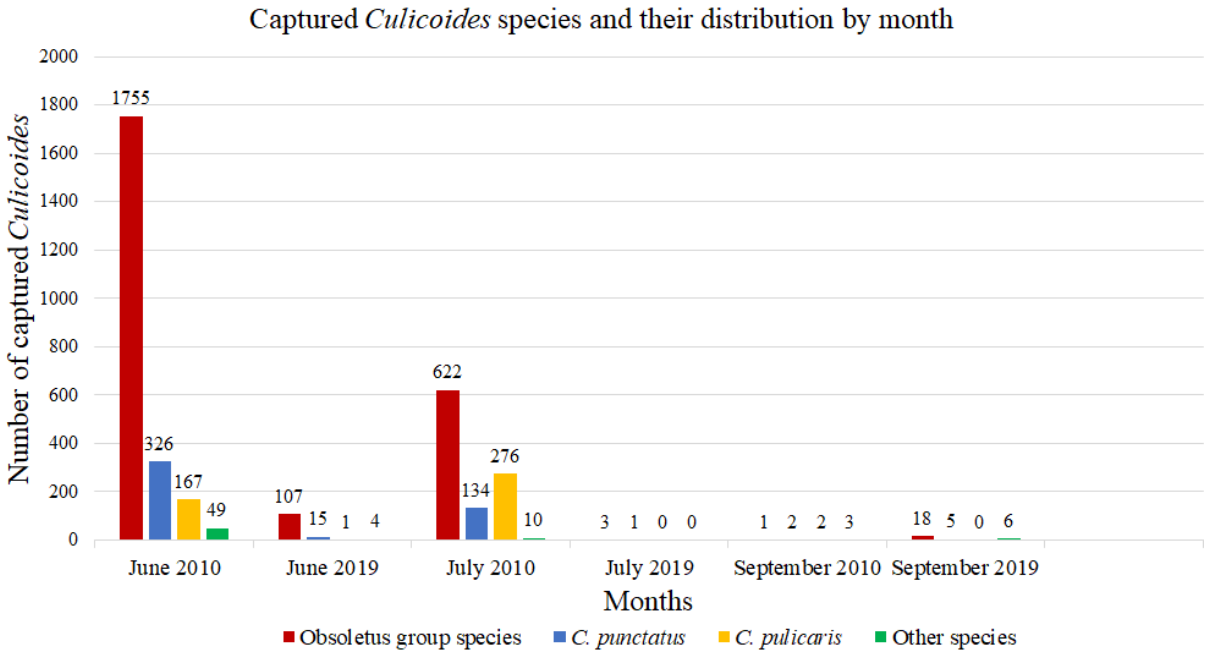


Figure 4.7: Captured *Culicoides* species and their distribution by month, between June and September (excluding August) 2010 and 2019.

In both years, June was clearly the month with more captured specimens with a total of 2297 and 127 *Culicoides* biting midges in 2010 and 2019, respectively. In this period of time, species from Obsoletus group represented the most caught species in both captures. In June 2010, Obsoletus group species represented 77% of all captures in that month. On the other hand, the same group species represented 84% of the total captured *Culicoides* of June 2019.

The following month was the second one with the most captured specimens in 2010, where Obsoletus group represented 60% of all the monthly captures. On the other hand, September was the second month where more specimens were caught in 2019 and Obsoletus group represented, once again, the major number of species captured in those traps, representing 62% of the total *Culicoides* caught.

4.3.3. Statistical Analysis: *Culicoides* species captured in Leiria and Lisbon (FMV-ULisbon)

For these tests, the presence of different *Culicoides* species was associated with the time they were captured. Thus, the null hypothesis (H_0) assumes that there are no association between the species captured in 2010 and species captured in 2019 and the alternative hypothesis (H_1) assumes that there are association between the species captured in 2010 and species captured in 2019.

From a total of 1251 *Culicoides* biting midges, the H_0 was:

- Chi-square test (*p*-value): 1.662e-27 (Rejected).
- Fisher's exact test (*p*-value): 0.0004998 (Rejected).

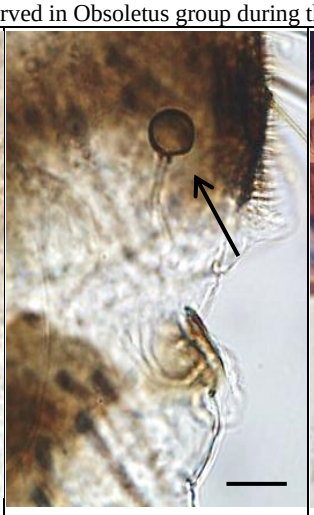
The overall statistical analysis showed that there is association between the distribution of *Culicoides* species and the time they were captured (which had a *p*-value below a pre-defined threshold of $p < 0.05$).

4.4. Morphological Anomalies found in Obsoletus group

During species identification, some abnormal morphological structures were found in specimens from Obsoletus group. These morphological alterations were observed in all collection sites.

The following aberrant anatomical aspects were observed: fused maxillary palp' articles, abdomen with the presence of one functional or non-functional spermathecae and three functional spermathecae instead of two functional and one rudimentary spermathecae. The morphological aberrations observed during this project in Obsoletus group are represented in table 4.5.

Table 4.5: Morphological aberrations observed in Obsoletus group during this project.

			
Scale bar: 16 µm	Scale bar: 17 µm	Scale bar: 17 µm	Scale bar: 14 µm
Specimen with 3 functional spermathecae	Specimen with one functional and another nonfunctional spermathecae	Specimen with no functional spermathecae	Fused maxillary palp' articles
Found in Leiria	Found in Leiria	Found in FMV-ULisbon	Found in LZ

4.4.1. Morphological anomalies found near Sylvatic Animals from Lisbon Zoo (LZ)

The aberrant anatomical aspects of species from Obsoletus group captured near sylvatic animals (giraffes and zebras) are described in table 4.6. No morphological aberrations were detected in *Culicoides* specimens captured near birds.

Table 4.6: Morphological aberrations per species, found in LZ.

Giraffes						
Morphological Anomalies		***Culicoides obsoletus***	**Total specimens with anomalies**	**Total specimens from Obsoletus group**	**Percentage of Anomalies**	**Total *Culicoides* captured**
3rd palpus segment	Fused maxillary palp' articles	1	1	50	2%	767

Table 4.6: Morphological aberrations per species, found in LZ. (Continuation)

Zebras						
Morphological Anomalies		***Culicoides obsoletus***	**Total specimens with anomalies**	**Total specimens from Obsoletus group**	**Percentage of Anomalies**	**Total *Culicoides* captured**
Spermathecae	3 functional spermathecae	1	1	61	1.64%	307

4.4.2. Morphological anomalies found domestic cattle from Leiria District

The aberrant anatomical aspects that were observed in *Culicoides* biting midges captured in Leiria District are described in table 4.7.

Table 4.7: Morphological aberrations per species, found in Leiria District.

Cattle (Old captures) – Leiria District							
Morphological Anomalies		***Culicoides obsoletus***	***Culicoides scoticus***	**Total specimens with anomalies**	**Total specimens from Obsoletus group**	**Percentage of Anomalies**	**Total *Culicoides* captured during NESP (2010)**
Spermathecae	3 functional spermathecae	1	1	3	106	2.83%	13391
	1 functional spermathecae	0	1				

4.4.3. Morphological anomalies found in domestic cattle from Faculty of Veterinary Medicine (FMV-ULisbon)

The aberrant anatomical aspects that were found in *Culicoides* biting midges captured in FMV-ULisbon are showed in table 4.8.

Table 4.8: Morphological aberrations per species, found in FMV-ULisbon.

Cattle (Recent captures) – FMV-ULisbon						
Morphological Anomalies		***Culicoides obsoletus***	**Total specimens with anomalies**	**Total specimens from Obsoletus group**	**Percentage of Anomalies**	**Total *Culicoides* captured**
3rd palpus segment	Fused maxillary palp' articles	3	8	128	6.25%	158
Spermathecae	3 functional spermathecae	4				
	1 nonfunctional spermathecae	1				

4.4.4. Molecular identification of morphological anomalies found in Obsoletus group species

From a total of 295 Obsoletus group specimens captured, 53 were used for molecular identification (including all *Culicoides* specimens with aberrant anatomical aspects from LZ and Leiria). However, during almost three months of several attempts, no results were obtained from PCR products of those *Culicoides* specimens with abnormal characteristics. The only obtained results were from *C. obsoletus* and *C. scoticus* species without aberrant aspects and since they supposed to be compared with the other specimens, they were not sent to STABvida. In Figure 4.8 there are presented some PCR products obtained during this project.

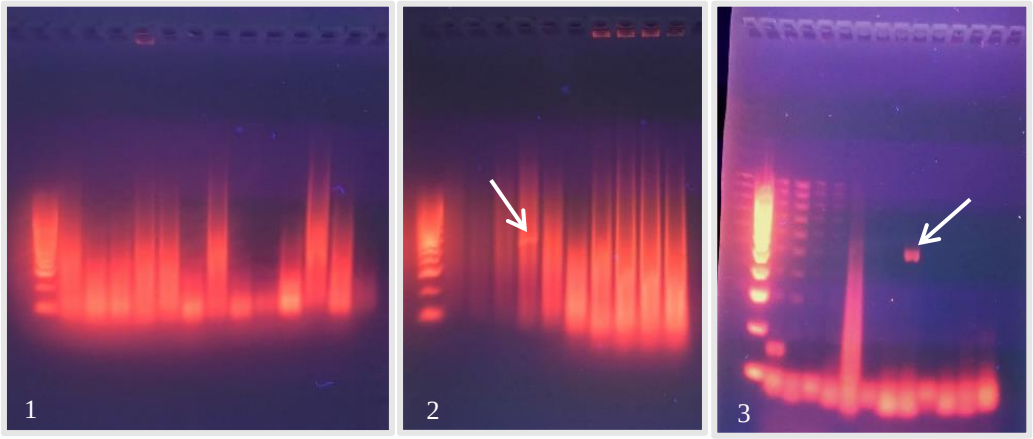


Figure 4.8: PCR products visualized in UV light.
1-Negative results – smeared bands; 2- Positive results – one band; 3- Positive results – one band.

5. DISCUSSION

5.1. *Culicoides* species captured near sylvatic animals and domestic cattle

Concerning vector-borne diseases, vectors are considered a relevant factor of an epidemiologic triad. Since they have an important role in the transmission of pathogen to a susceptible host, a deeper knowledge of the vector and the factors that control their appearance, development, prevalence and death are necessary to better understand the dynamics of VBD. Analyzing *Culicoides* fauna present in a specific area, their distribution near hosts, seasonal occurrence and other factors have a major importance, since this information can contribute to understand the process by which a disease appears, maintains and spreads from an area to another. Besides that, it is well known that causative agents of several diseases are shared between wildlife and livestock, and it is important to understand that some *Culicoides* species can act as a bridge vectors and transfer some viruses from domestic to wild ruminant populations since domestic animals are important factors for attracting and maintaining biting midge populations in peridomiciliary areas [82].

Previous studies on *Culicoides* fauna were made in Portugal [9, 73, 83], however, there has never been made a study of these biting midges near sylvatic animals and their comparison with domestic cattle. Nevertheless, similar entomological studies have been carried out in farms and forest reserves in Europe [84, 85, 86] and Brazil [82].

In this study a relative high number of different *Culicoides* species have been captured near sylvatic animals (N=135; ($\bar{x}$)= 16.88 per trap; 8 different species) and some of these species were also present near domestic cattle (N=158; ($\bar{x}$)= 22.57 per trap; 7 different species), between June and September 2019 (excluding August). However, in that same period, more specimens were captured in FMV-ULisbon than in LZ. The majority of *Culicoides* species caught is considered mammal-feeders (mammalophilic) as expected, since most of the possible hosts are mammals. Nevertheless, some studies have shown that these biting midges can be opportunist feeders, with some species previously considered as ornithophilic or indefinite feeders detected as mammal feeders, using molecular bloodmeal analysis [87, 88].

The most abundant species caught near sylvatic animals were *C. imicola* (70%), *C. parroti* (12%) and *C. nubeculosus* (7%) and near domestic cattle were *C. obsoletus* (54%), *C. scoticus* (27%) and *C. punctatus* (13%). Similar results were presented in a study made in Czech Republic, where from almost half million biting midge specimens collected during five years from domestic livestock and wild ruminants from forest parks, the Obsoletus group species represented 91% and 52% of all captures, respectively, and emphasize the potentially high vector capacity of these species. On the other hand, different results were obtained in another similar study made in Spain [84] describing *C. imicola* as more abundant at livestock farms than at natural areas, while Obsoletus group species were caught more easily near wild animals.

These results could be explained by the particular environment and conditions where the traps were placed, such as availability of breeding sites, surrounding environment and, also, depends on geographical areas (e.g., latitude). Concerning breeding sites preferences, *C. imicola* breeds in moisture retentive soils enriched with organic material while *C. obsoletus* prefers cattle dung. In both sites a cleaning service was performed daily, however more cattle dung was present near cows from FMV-ULisbon, which allows the presence of appropriate breeding sites [85]. Also, *Culicoides* species composition at local sites with sylvatic animals could be influenced by the surrounding environment (e.g., additional suitable hosts or bushes) and occasional presence of hosts, since they can move away from the traps, in contrast to farms, where livestock is placed in stables and almost continuously in the proximity of traps [89]. Additionally, supporting these results, the statistical analysis confirmed that the composition of *Culicoides* species depends on the local where they were captured.

This study showed that the main BTV vectors in Europe, *C. imicola* and Obsoletus group species, are present in LZ and FMV-ULisbon, respectively, which would support their putative role as bridge vectors for the transmission of the arboviruses between sylvatic animals and domestic cattle and once introduced, consequently, will spread rapidly over large regions of Europe when appropriate environmental conditions and hosts are present.

5.2. *Culicoides* species captured near sylvatic animals: birds, giraffes and zebras

The monitorization of *Culicoides* biting midges in zoo environment is important to avoid potential risk of outbreaks since imported animals could be reservoirs of diseases and zoo is a place where a large number of potential vectors may be present. In 1987 in Spain, this situation happened via the importation of zebras from Namibia to a wildlife park near Madrid, where *C. imicola* was present and allowed an outbreak of AHSV [90]. Also, there are several concerns about the spread of diseases in a zoo environment, since sylvatic animals have a very high value, both financial and as part of international breeding programs for species conservation, that could be seriously affected if animals get sick.

In this study, the quantitative and qualitative composition of the captures made between May 2018 and September 2019 near birds, giraffes and zebras was compared, where, from a total of *Culicoides* specimens captured near each animal species, birds (N=14; ($\bar{x}$)= 2.33 per trap; 6 different species), giraffes (N=767; ($\bar{x}$)= 47.94 per trap; 16 different species) and zebras (N=307; ($\bar{x}$)= 76.75 per trap; 5 different species), it is noticeable that giraffes had a major number of specimens captured nearby but, on the other hand, zebras had a bigger amount of *Culicoides* biting midges captured per trap. These results suggest that more *Culicoides* species may have a preference for giraffes, which indicates that these hosts are more attractive than zebras and birds. This has an important epidemiological implication since zebras could be a susceptible target for these vectors when giraffes are absent in the vicinity. Similar results were suggested in a previous study made in Whipsnade Zoo, where giraffes allow the collection of the most individuals in a single night and zebras are unlikely to have an epidemiological significance, but there may be a risk associated due to their role as reservoir hosts and the importation of these animals [91].

Also, in this study, the samples of biting midge faunas in LZ collected near giraffes and zebras had as most abundant species *C. imicola*, with 77% and 80%, and *C. obsoletus* as the second most abundant, with 6% and 17%, respectively. Near birds, *C. obsoletus* represented 57% of the *Culicoides* specimens captured. Since most of the captured *Culicoides* are vectors of BTV, with *C. imicola* being the primary vector of BTV and AHSV [92], if they reach a significant density in LZ and if BTV and/ or AHSV is also present there would probably be a high risk of transmission and spread of those diseases. However, from a previous study made in Chester Zoo [93], Obsoletus group represented the largest part of the captures, with over 94% of all the female *Culicoides* trapped. These differences could be explained since, according to Fauna Europaea (2020) [20], in the UK *C. imicola* is absent.

Furthermore, captures made near birds represented an unexpected result in birdfeeders (ornithophilic) *Culicoides* species, with just one species being captured, *C. circumscriptus*, and being the mammal-feeders, *C. obsoletus* and *C. gejgelensis* the most captured ones, with 57% and 15%, respectively. Besides that, *C. circumscriptus* is capable of feed on a large variety of mammals, that is why is also found near giraffes, since it has a wide adaptability to different environmental conditions. More attention must be given to this species, since a recent study suggests that this species is a potential vector of BTV [94]. Some factors can influence biting midges original feeding pattern, such as the appearance of secondary hosts [85], but a major part of *Culicoides* species have some flexibility in host selection and members of Obsoletus group feed primarily on mammals and only occasionally on birds, being this feeding pattern supported by some previous studies [87, 95, 96].

Additionally, there were statistically significant association between the distribution of *Culicoides* species and the animal type near where the trap was placed, likely due to a range of factors such as the level of wind exposure, potential hosts proximity and exposure, the relative density and size of hosts surrounding the trap and the availability of larval habitats close to the traps.

5.3. *Culicoides* species captured near domestic cattle from Leiria and Faculty of Veterinary Medicine (FMV-ULisbon)

In this study, the quantitative and qualitative composition of the captures made in the period between June and September of 2010 and 2019 (excluding August on both years) was compared.

A relative high number of different *Culicoides* species were captured in Leiria and several of those species also appeared in FMV-ULisbon more recently. In Leiria, some of the captured species were not present in the most recent captures, such as *C. indistinctus* Khalaf, 1961, *C. fascipennis* (Staeger, 1839), *C. univitattus*, *C. heteroclitus* Kremer & Callot, 1965 and *C. achrayi* Kettle & Lawson, 1955, while in FMV-ULisbon only one different species appeared, *C. paolae*. Although there were limitations, as time and different collection sites, usually *Culicoides* species absence/ presence is affected by abiotic and biotic factors, e.g. climatic variables, types of vegetation and soil, presence of water courses, hosts, and others, depending on the species preferences [9].

According to Fauna Europaea and Bruno Mathieu and collaborators, *C. paolae* was only present in Italy area, Corsica, Sardinia and Crete, Greece [18, 25]. Furthermore, the first report of this species in Portugal occurred in 2003 [83]. Recently, a study was carried out in Sardinia, Italy where *C. paolae* was considered as a possible BTV vector, which means they could play an important role in the transmission between natural reservoir hosts and domestic ruminants [14]. Also, other authors reported this species to be among the most abundant species in cattle farms in Mediterranean area [97, 98].

It is well known that the long persistence of some *Culicoides* species occur due to several factors, being climate the most influent in the dynamics of these midges, and climate change affects them by increasing the minimum winter temperatures and the annual precipitation that will influence the distribution of the species (e.g. the availability of moist breeding sites), as well the duration of seasonal vector-free period [99]. According to *Instituto Português do Mar e da Atmosfera* (IPMA) in 2010, four heat waves occurred in mainland Portugal, one in May, two in July and one in August and two of them coincide with the period of the captures that were made for this study [100]. Since modern recordkeeping began in 1880, the year 2019 was the second warmest on record after 2016, where the average temperatures for the ten-year (2010-2019) period were the highest registered. Furthermore, since the 1980s each decade has been warmer than the previous one [101]. Particularly, in 2019, four heat waves were reported in non-summertime, one of those was in September. On the other hand, June was considered the coldest since the beginning of the XXI century [102].

Comparing the total number of captures, Leiria 2010 (N=3347; ($\bar{x}$)= 418.4 per trap; besides Obsoletus group, 7 different species were found) and FMV-ULisbon 2019 (N=158; ($\bar{x}$)= 26.33 per trap; besides Obsoletus group, 5 different species were found), it is noticeable that occurred a fall in *Culicoides* species captured and this may have been caused by the hot and dry summer in this year, which may cause a deficit in the creation of favourable conditions for *Culicoides* species. Regarding the proportion of the most abundant species during these periods, the results showed that, in 2010, Obsoletus group species represented 71% of all the captures, *C. punctatus*, 14% and *C. pulicaris*, 13%. Also, in 2019, Obsoletus group species represented the majority of all captures with 81%, followed by *C. punctatus* with 13% and *C. imicola* with 3%. Species from Obsoletus group are the most abundant and widespread species in both captures, increasing from 71% to 81% in composition, which is important to notice, since those species can acquire, maintain and spread BTV virus.

In regions above Tagus River, Obsoletus group species population are more common during spring and summer time. Besides their occurrence is favoured by lower mean temperatures during the driest quarter of spring and winter seasons. However, they seemed to be limited by dryness and usually declined in summer dry months [9]. The year 2019 was characterized for having an extended dry season, from January to October, and, also, the four heat waves did not occur in summertime [102]. When compared to 2010, these conditions may be the reason for the drop in the number of captured *Culicoides*, since this modification can be an impediment for larvae development and reduce breeding sites [9].

Species captured at the year 2010, such as *C. pulicaris*, were not significatively active throughout the year 2019, probably because of the reduction of their breeding sites at FMV-ULisbon that could limit their expansion to this location. Although *C. pulicaris* is present during all year, it is more common during spring season and water courses raise the probability of their appearance during summer since larvae development requires stagnant water [9]. *C. punctatus* has the probability of 50% or more to occur in any part of mainland Portugal in almost all seasons, being very well adapted to our climate and, although *C. punctatus* larvae have been found in the same places as *C. pulicaris*, they are more adapted to our edaphoclimatic environment [9]. Plus *C. imicola*, the major vector of BTV, was not captured in larger quantities during this period since is more common in regions bellow Tagus River from spring to fall. Besides, it is also limited by dry stress and to a lesser extent wet stress [9, 103]. Additionally, there were statistically significant association between the distribution of *Culicoides* species and the time they were captured.

Furthermore, it was made a detailed analysis of the species distribution during these three months, where June was pointed as the month where more specimens were captured in both years.

The seasonal dynamic of *C. obsoletus*/*C. scoticus* has a characteristic pattern, where the first *Culicoides* specimens of these species appear in April, peak between May and June, lowering their abundance during July and it is followed by a higher abundance during August and September [104]. These species can also be caught at low temperatures which may affect the potential for BTV transmission during cold seasons [9].

The occurrence of *C. punctatus* in these regions is equally distributed during all year and, as expected, *C. pulicaris* reached its population peak one month later, in July, since these biting midges are associated with warmer temperatures [9, 89].

A previous study showed that species from Obsoletus group occur in northern Europe (The Netherlands and Sweden) in relatively high proportions. The capacity of these species to acquire viruses (e.g. BTV) in combination with their wide distribution and high densities at livestock farms, make them, likely candidates for rapid spread of midge-borne viruses throughout Europe [105].

5.4. Morphological Anomalies in *Culicoides* from Obsoletus group

It has been suggested that *C. obsoletus* and *C. scoticus* are adapted to a wider range of habitats and are more resistant to extreme low temperatures [106] and the availability of suitable hosts (horses, cattle, sheep, goats) for female biting midges, or larval breeding sites (e.g., dung, edges of ponds, marshes, tree holes) could influence the relative abundances of species found [107]. Since Obsoletus group species are very similar, the crossbreeding between close related species can result in genetic errors that could cause morphological aberrations [25], and some of them have already been referred in previous studies [31, 108, 109] including *C. obsoletus* with three functional spermathecae [32].

From the total of *Culicoides* from Obsoletus group captured, FMV-ULisbon represented the place where specimens had more anatomical anomalies in the total of specimens captured, with 6.25%, followed by Leiria with 2.83%. From the captures near sylvatic animals those caught near giraffes presented more anatomical malformations, with 2%. During this project, morphological anomalies were found in *C. obsoletus* and *C. scoticus* from all collection sites: maxillary palps with fused articles and

specimens with one or three spermathecae. From the two species, *C. obsoletus* was the one with more anomalies observed during this study.

The referred modifications were observed in mounted specimens because they are compatible with midge survival and thus maintained in adult specimens. On the other hand, *Culicoides* with anatomical changes not compatible with survival do not reach the adult phase or do not live long enough to be captured with light traps. These alterations observed in *Culicoides* are important, since they deposit 10 to 675 eggs, depending on species, so there is a high probability for anatomical aberrations to occur due to genetic or morphogenetic malformation [27, 110]. Also, these anomalies in structures with specific functions can possibly affect insects' life activities [110]. However, the report of different types of anatomical aberrations is important, since these morphological alterations can lead to incorrect species identification during sample analysis.

Unfortunately, molecular analysis of these specimens with abnormal characteristics were not possible to be discussed since during the observation in UV light of PCR products, instead of having well-defined bands containing a large number of DNA fragments of the same size, the smeared bands appeared. These smeared bands are not very useful since contains many different sizes of DNA and the molecules running together probably have nothing in common. They can appear due several reasons, being usually the result of poorly prepared gels, loading undiluted samples into the wells or poor-quality samples [111]. In order to avoid the appearance of smear bands some procedures were made. Concerning the DNA quantity and quality of specimens, the samples were diluted enough to run through the gel without overflowing the wells and their purity was tested in order to detect contamination by proteins. Besides that, freezing and thawing DNA samples repeatedly and the degradation caused by the years of samples from Leiria could affect their quality. However, after all the efforts and several attempts, no results were obtained.

5.5. Study limitations

Some limitations happened during this project, such as the comparison of domestic cattle (FMV-ULisbon) and sylvatic animals (LZ), where the location was not the same due to the domestic cattle enclosures in LZ, since those were very exposed to visitors that could interfere with the trap and influence the capture results (e.g. steal or damage the trap, as already happened in previous experiments). However, the traps were placed in the same city.

The same limitation appears when the comparison of old captures (Leiria) with the most recent ones (FMV-ULisbon) where the location is also different. However, that occurred because it was impracticable to go to Leiria every month, attending the project time to make the captures and because those old captures had authorization and financial support during NESP. The data of these old captures was, primarily, used because of the presence of female midges with unique aberrant anatomical aspects that the author wanted to evaluate for a better understanding of their presence.

Another limitation that can be pointed out is the fact that the performed captures can influence the total number of *Culicoides* specimens caught. So, it is important to notice that LZ and Leiria captures were made for other projects, with different purposes. When those sites are compared to FMV-ULisbon, it is important to understand that a quantitative discrepancy in the number of *Culicoides* captured exists. Besides that, in FMV-ULisbon, one of the captures made in June did not caught any *Culicoides* species but it was considered in this project results since there was not any functional problem with the trap. On the other hand, in July there were technical issues with the trap' functions, affecting the correct capture of midges, resulting in one less capture made that month. Although the differences in the number of captures, they do not significantly undermine the results.

6. CONCLUSIONS

6.1. Main Conclusions

The present work allowed the collection of data concerning *Culicoides* species captured in three different sites: LZ, Leiria and FMV-ULisbon, for the analysis of *Culicoides* fauna near these sites, showing their frequency and which species were present near different animals (domestic and wild).

It is well known that causative agents of several diseases are shared between wildlife and livestock, and it is important to understand that some *Culicoides* species can act as a bridge vectors and transfer some viruses from domestic to wild ruminant populations since domestic animals are important factors for attracting and maintaining biting midge populations in peridomiciliary areas.

Furthermore, there are several concerns about the spread of diseases between wild animals in a zoo environment and since the abundance of *Culicoides* species in these locations reveals that the main BTV vectors in Europe, *C. imicola* and Obsoletus group species (*C. obsoletus* and *C. scoticus*), are the most prevalent ones, we must considerate a probably high risk of transmission and spread. When comparing the 9-year period in farm environments, is important to notice that a drop in captured *Culicoides* species occurred, probably caused by the hot and dry year of 2019, which may cause a deficit in the creation of favourable conditions for *Culicoides* species development. Furthermore, once again, Obsoletus group species takes 71% and 81% of all the captures in 2010 and 2019, respectively.

Since species inside Obsoletus group are very similar, the crossbreeding of close related species can result in genetic errors that could cause morphological modifications and this project gives essential information concerning these aberrant characteristics, including a specimen with only one non-functional spermathecae in *C. obsoletus*, which was never reported before to the best of our knowledge. Furthermore, a molecular analysis of these specimens with anatomical aberrations was made in order to understand their taxonomical position but, after several attempts, no results were obtained.

C. imicola and species from Obsoletus group (*C. obsoletus* and *C. scoticus*), known as BTV vectors in Europe, were the most captured species during this project. Their distribution and abundance are affected by abiotic factors, such as climate, temperature, wind exposure, soil type, surrounding vegetation and other factors, such as the potential host proximity and exposure, density and size of hosts surrounding the trap and the availability of larval habitats close to the traps. There is a concern that climate change will lead to expansion of VBD's, since insects are sensitive to those factors. These VBD's affect tens of thousands of farms causing huge financial costs and the death of millions of animals. Such expansion may also threaten human health, and food security due to their effects on animal and crop health. The creation of methods to accurate disease detection and efficient strategies for control of high-risk population movements from infected areas will be essential to minimize the impact of BTV and similar newly emerging VBDs in a future warmer world.

6.2. Future Perspectives

Further studies should be carried out in order to support the results obtained in this project, such as understand how different variables influence the captures near hosts (sylvatic and domestic) and their occurrence in different seasons because it would contribute for understanding these biting midges dynamics and would be an extremely important auxiliary information to further studies concerning ecological preferences and control measures that must be taken in order to reduce the risk of VBD and other diseases outbreaks. Also, an evaluation of geographical areas where major vectors are present should be carried, as well as the identification of considered "non-vectors" species, since they can be also involved in the transmission.

Since the distribution of these biting midges is strongly influenced by the existence of different breeding sites, a study should be carried out on the specific physiological or behavioural adaptations of immature stages of *Culicoides* midges, which might be an important key to explain the differences in the breeding site selection between different *Culicoides* species. Plus, a molecular identification of the blood meal origin from blood fed *Culicoides* should be conducted to better understand their host preferences.

Concerning the observed specimens that possess intermediary characteristic or anatomical malformations, further studies must be performed and compared with known *Culicoides* species in order to understand their actual taxonomic position. Since works concerning these morphological alterations in *Culicoides* specimens are scarce, further studies of these descriptions can help with the correct species identification.

7. BIBLIOGRAPHY

[1] WHO | World Health Organization (2019). *Vector-borne diseases*. Accessed on the 8th September, 2019, available in: https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases

[2] Barker, C. M., & Reisen, W. K. (2019). Epidemiology of Vector-Borne Diseases. In *Medical and Veterinary Entomology*. Academic Press, 33-49.

[3] Chippaux, A. (2003). Généralités sur arbovirus et arboviroses. *Médecine et maladies infectieuses*, *33*(8), 377-384.

[4] OIE | World Organization for Animal Health (2020). OIE – Listed diseases, infections and infestations in force in 2020. Updated on the 1st January 2020. Accessed on the 4th January, 2020, available in: https://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2020

[5] Slama, D., Babba, H., & Chaker, E. (2017). *Culicoides* spp. (Diptera: Ceratopogonidae) in Tunisia. In *Biological Control of Pest and Vector Insects*. IntechOpen, 213-219.

[6] Mellor, P. S., Boorman, J., & Baylis, M. (2000). *Culicoides* biting midges: their role as arbovirus vectors. *Annual review of entomology*, *45*(1), 307-340.

[7] Carpenter, S., Groschup, M. H., Garros, C., Felippe-Bauer, M. L., & Purse, B. V. (2013). *Culicoides* biting midges, arboviruses and public health in Europe. *Antiviral research*, *100*(1), 102-113.

[8] Borkent, A. (2005). The biting midges, the Ceratopogonidae (Diptera). *Biology of diseases vectors*, 113-126.

[9] Ramilo, D.W. (2016). Phenotypic and genetic characterization of *Culicoides* (Diptera: Ceratopogonidae) in Portugal and comparison of the effect of pyrethroid insecticides in their control. *Ph.D. Thesis in Veterinary Sciences, Animal Health. Universidade de Lisboa, Faculdade de Medicina Veterinária, Lisboa*.

[10] Mellor, P. S., & Hamblin, C. (2004). African horse sickness. *Veterinary research*, *35*(4), 445-466.

[11] Yanase, T., Kato, T., Kubo, T., Yoshida, K., Ohashi, S., Yamakawa, M., Miura, Y., & Tsuda, T. (2005). Isolation of bovine arboviruses from *Culicoides* biting midges (Diptera: Ceratopogonidae) in southern Japan: 1985-2002. *Journal of Medical Entomology, 42(1)*, 63-67.

[12] Sick, F., Beer, M., Kampen, H., & Wernike, K. (2019). *Culicoides* Biting Midges—Underestimated Vectors for Arboviruses of Public Health and Veterinary Importance. *Viruses*, *11*(4), 376.

[13] Goffredo, M., Catalani, M., Federici, V., Portanti, O., Marini, V., Mancini, G., Quaglia, M., Santilli, A., Teodori, L., & Savini, G. (2015). Vector species of *Culicoides* midges implicated in the 2012–2014 Bluetongue epidemics in Italy. *Veterinaria Italiana*, *51*(2), 131-8.

[14] Foxi, C., Meloni, G., Puggioni, G., Manunta, D., Rocchigiani, A., Vento, L., Cabras, P., & Satta, G. (2019). Bluetongue virus detection in new *Culicoides* species in Sardinia, Italy. *Veterinary Record*, *184*(20), 621-621.

[15] Gordon, S.J., Bolwell, C., Rogers, C., Musuka, G., Kelly, P., Labuschagne, K., Guthrie, A.J., Denison, E., Mellor, P.S., & Hamblin, C. (2015). The occurrence of *Culicoides* species, the vectors of arboviruses, at selected trap sites in Zimbabwe. *Onderstepoort Journal of Veterinary Research*, *82*(1), 01-08.

[16] Dik, B., Yavru, S., Uslu, U., Yapıcı, O., & Esin, E. (2012). Determination of *Culicoides* species (Diptera: Ceratopogonidae) as suspect vectors of epizootic haemorrhagic disease and bluetongue viruses in southern and western Anatolia by RT-PCR. *Revue de Médecine Vétérinaire*, *163*(11), 505-510.

[17] Mellor, P. S., Boorman, J., & Loke, R. (1974). The multiplication of Main Drain virus in two species of *Culicoides* (Diptera, Ceratopogonidae). *Archiv für die gesamte Virusforschung*, *46*(1-2), 105-110.

[18] De Regge, N., Deblauwe, I., De Deken, R., Vantieghem, P., Madder, M., Geysen, D., Smeets, F., Losson, B., van den Berg, T., & Cay, A. B. (2012). Detection of Schmallenberg virus in different *Culicoides* spp. by real-time RT-PCR. *Transboundary and emerging diseases*, *59*(6), 471-475.

[19] Collins, Á. B., Doherty, M. L., Barrett, D. J., & Mee, J. F. (2019). Schmallenberg virus: a systematic international literature review (2011-2019) from an Irish perspective. *Irish Veterinary Journal*, *72*(1), 9.

[20] Fauna Europaea (2019). Taxonomic Hierarchy. Accessed on the 8th September, 2019, available in: https://fauna-eu.org/cdm_dataportal/taxon/52399942-cec0-4461-952e-3dc4a4f4cdfa

[21] Borkent, A. (2016). Numbers of extant and fossil species of Ceratopogonidae. Accessed on the 8th September, 2019, available in: https://www.inhs.illinois.edu/files/4014/6785/5847/WorldCatalogtaxa.pdf
[22] Borkent, A. (2014). The pupae of the biting midges of the world (Diptera: Ceratopogonidae), with a generic key and analysis of the phylogenetic relationships between genera. *Zootaxa*, *3879*(1), 1-327.
[23] Harrup, L. E., Bellis, G. A., Balenghien, T., & Garros, C. (2015). *Culicoides* Latreille (Diptera: Ceratopogonidae) taxonomy: current challenges and future directions. *Infection, Genetics and Evolution*, *30*(1), 249-266.
[24] Purse, B. V., Carpenter, S., Venter, G. J., Bellis, G., & Mullens, B. A. (2015). Bionomics of temperate and tropical *Culicoides* midges: knowledge gaps and consequences for transmission of *Culicoides*-borne viruses. *Annual review of entomology*, *60*(1), 373-392.
[25] Mathieu, B., Cêtre-Sossah, C., Garros, C., Chavernac, D., Balenghien, T., Carpenter, S., Setier-Rio, M. L., Vignes-Lebbe, R., Ung, U., Candolfi, E., Delécolle, J. C. (2012) Development and validation of IIKC: an interactive identification key for *Culicoides* (Diptera: Ceratopogonidae) females from the Western Palearctic region. *Parasites & Vectors*, *5*(1), 137.
[26] Wirth, W. W., & Hubert, A. A. (1989). The *Culicoides* of Southeast Asia (Diptera: Ceratopogonidae). *Walter Reed Army Inst of Research Washington DC*, *44*(1), 508.
[27] González, M., & Goldarazena, A. (2011). Género *Culicoides* en el País Vasco. *Eusko Jaurlaritzaren Argitalpen Zerbitzu Nagusia, Servicio Central de Publicaciones del Gobierno Vasco*, 247.
[28] Delécolle, J.C. (1985). Nouvelle contribution à l'étude systématique et iconographique des espèces du genre *Culicoides* (Diptera: Ceratopogonidae) du nord-est de la France. *Thèse de doctorat, U.E.R. Sciences Vie et Terre. Strasbourg: Université Louis Pasteur*.
[29] Pagès, N., & Sarto I Monteys, V. (2005). Differentiation of *Culicoides obsoletus* and *Culicoides scoticus* (Diptera: Ceratopogonidae) based on mitochondrial cytochrome oxidase subunit I. *Journal of Medical Entomology*, *42(6)*, 1026-1034.
[30] Ramilo, D., Garros, C., Mathieu, B., Benedet, C., Allène, X., Silva, E., Alexandre-Pires, G., Pereira da Fonseca, I., Carpenter, S., Rádrová, J., & Delécolle, J-C. (2013). Description of *Culicoides paradoxalis* sp. nov. from France and Portugal (Diptera: Ceratopogonidae). *Zootaxa*, *3745(2)*, 243-256.
[31] Callot, J., & Kremer, M. (1963). Intersexués chez des *Culicoides* (Diptera: Ceratopogonidae). *Annales de parasitologie humaine et comparee*, *38*(1), 113-120.
[32] McKEEVER, S. T. U. R. G. I. S., Brickle, D. S., & Hagan, D. V. (1997). Mouthparts, antennae and genitalia of intersex *Culicoides stellifer* parasitized by mermithid nematodes. *Medical and veterinary entomology*, *11*(3), 217-222.
[33] Purse, B. V., Mellor, P. S., Rogers, D. J., Samuel, A. R., Mertens, P. P., & Baylis, M. (2005). Climate change and the recent emergence of bluetongue in Europe. *Nature Reviews Microbiology*, *3*(2), 171-181.
[34] Morag, N., Mullens, B. A., & Gottlieb, Y. (2013). Assessment of survival and body size variation of *Culicoides imicola* (Diptera: Ceratopogonidae) as functions of "*Candidatus* Cardinium"(Bacteroidetes) infection status. *Applied and Environmental Microbiology*, *79*(20), 6260-6263.
[35] Lysyk, T.J. (2007). Seasonal abundance, parity, and survival of adult *Culicoides sonorensis* (Diptera: Ceratopogonidae) in southern Alberta, Canada. *Journal of Medical Entomology*, *44*(6), 959-969.
[36] Lassen, S. B., Nielsen, S. A., & Kristensen, M. (2012). Identity and diversity of blood meal hosts of biting midges (Diptera: Ceratopogonidae: *Culicoides* Latreille) in Denmark. *Parasites & vectors*, *5*(1), 143.
[37] Blackwell, A. (2004). A morphological investigation of *Culicoides* spp. biting midges (Diptera: Ceratopogonidae) from the Caribbean. *Journal of Vector Ecology*, *29*(1), 51-61.
[38] Rossi, S., Balenghien, T., Viarouge, C., Faure, E., Zanella, G., Sailleau, C., C., Mathieu, B., Delécolle, J.C., Ninio, C., Garros, C., & Gardès, L. (2019). Red deer (Cervus elaphus) did not play the role of maintenance host for bluetongue virus in France: The burden of proof by long-term wildlife monitoring and *Culicoides* snapshots. *Viruses*, *11*(10), 903.
[39] Leta, S., Fetene, E., Mulatu, T., Amenu, K., Jaleta, M. B., Beyene, T. J., Negussie, H., & Revie, C. W. (2019). Modeling the global distribution of *Culicoides imicola*: an Ensemble approach. *Scientific reports*, *9*(1), 1-9.

[40] Gutsche, T. (1979). There was a man: the life and times of Sir Arnold Theiler, KCMG, of *Onderstepoort*, 4. Cape Town: Howard Timmins.
[41] Hutcheon, D. (1881). Contagious pleuro-pneumonia in angora goats. *The Veterinary Journal and Annals of Comparative pathology*, *13*(9), 171-180.
[42] Spreull, J. (1905). Malarial catarrhal fever (bluetongue) of sheep in South Africa. *Journal of Comparative Pathology and Therapeutics*, *18*(1), 321-337.
[43] MacLachlan, N. J. (2004). Bluetongue: pathogenesis and duration of viraemia. *Veterinaria Italiana*, *40*(4), 462-7.
[44] Rodríguez-Sánchez, B., Iglesias-Martín, I., Martínez-Avilés, M., & Sánchez-Vizcaíno, J. M. (2008). Orbiviruses in the Mediterranean basin: updated epidemiological situation of Bluetongue and new methods for the detection of BTV serotype 4. *Transboundary and emerging diseases*, *55*(5-6), 205-214.
[45] Shimshony, A. (2004). Bluetongue in Israel–a brief historical overview. *Veterinaria Italiana*, *40*(3), 116-8.
[46] Hardy, W. T., & Price, D. A. (1952). Soremuzzle of sheep. *Journal of the American Veterinary Medical Association*, *120*(898), 23.
[47] Gibbs, E. P. J., & Greiner, E. C. (1994). The epidemiology of bluetongue. *Comparative immunology, microbiology and infectious diseases*, *17*(3-4), 207-220.
[48] Clavijo, A., Sepulveda, L., Riva, J., Pessoa-Silva, M., Tailor-Ruthes, A., & Lopez, J. W. (2002). Isolation of bluetongue virus serotype 12 from an outbreak of the disease in South America. *Veterinary record*, *151*(10), 301-301.
[49] Mellor, P. S., Carpenter, S., Harrup, L., Baylis, M., & Mertens, P. P. (2008). Bluetongue in Europe and the Mediterranean Basin: history of occurrence prior to 2006. *Preventive veterinary medicine*, *87*(1-2), 4-20.
[50] Mellor, P. S., & Wittmann, E. J. (2002). Bluetongue virus in the Mediterranean Basin 1998–2001. *The Veterinary Journal*, *164*(1), 20-37.
[51] Carpenter, S., Wilson, A., & Mellor, P. S. (2009). *Culicoides* and the emergence of bluetongue virus in northern Europe. *Trends in microbiology*, *17*(4), 172-178.
[52] OIE | World Organization for Animal Health (2019). OIE – Animal Diseases: Bluetongue. Accessed on the 1st December 2019, available in: https://www.oie.int/en/animal-health-in-the-world/animal-diseases/bluetongue/
[53] Wilson, A. J., & Mellor, P. S. (2009). Bluetongue in Europe: past, present and future. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1530), 2669-2681.
[54] Bumbarov, V., Golender, N., Jenckel, M., Wernike, K., Beer, M., Khinich, E., Zalesky, O., & Erster, O. (2020). Characterization of bluetongue virus serotype 28. *Transboundary and emerging diseases*, *67*(1), 171-182.
[55] Elbers, A. R., Backx, A., Meroc, E., Gerbier, G., Staubach, C., Hendrickx, G., Van der Spek, A., & Mintiens, K. (2008). Field observations during the bluetongue serotype 8 epidemic in 2006: I. Detection of first outbreaks and clinical signs in sheep and cattle in Belgium, France and the Netherlands. *Preventive veterinary medicine*, *87*(1-2), 21-30.
[56] Meiswinkel, R., Gomulski, L. M., Delécolle, J. C., Goffredo, M., & Gasperi, G. (2004). The taxonomy of *Culicoides* vector complexes–unfinished business. *Veterinaria Italiana*, *40*(3), 151-159.
[57] Beerntsen, B. T., James, A. A., & Christensen, B. M. (2000). Genetics of mosquito vector competence. *Microbiology and Molecular Biology Reviews*, *64*(1), 115-137.
[58] Van Dijk, A. A., & Huismans, H. (1982). The effect of temperature on the in vitro transcriptase reaction of bluetongue virus, epizootic haemorrhagic disease virus and African horsesickness virus. *Onderstepoort Journal of Veterinary Research*, *49*(1), 227-232.
[59] Müller, U., Kemmerling, K., Straet, D., Janowitz, U., Sauerwein, H. (2010). Effects of Bluetongue virus infection on sperm quality in bulls: a preliminary report. *The Veterinary Journal*, *186*(1), 402-403.
[60] Vanbinst, T., Vandenbussche, F., Dernelle, E., & De Clercq, K. (2010). A duplex real-time RT-PCR for the detection of bluetongue virus in bovine semen. *Journal of virological methods*, *169*(1), 162-168.

[61] Breard, E., Pozzi, N., Sailleau, C., Durand, B., Catinot, V., Sellem, E., P. Dumont, B. Guerin & Zientara, S. (2007). Transient adverse effects of an attenuated bluetongue virus vaccine on the quality of ram semen. *Veterinary Record*, *160*(13), 431-435.
[62] Van der Sluijs, M. T. W., Schroer-Joosten, D. P. H., Fid-Fourkour, A., Smit, M. A. O., Vrijenhoek, M. P., Moulin, V., A. J. de Smit & Moormann, R. J. M. (2013). Transplacental transmission of BTV-8 in sheep: BTV viraemia, antibody responses and vaccine efficacy in lambs infected in utero. *Vaccine*, *31*(36), 3726-3731.
[63] Katz, J., Alstad, D., Gustafson, G., Evermann, J. (1994). Diagnostic analysis of the prolonged bluetongue virus RNA presence found in the blood of naturally infected cattle and experimentally infected sheep. *Journal of Veterinary Diagnostic Investigation*, *6*(1),139-142.
[64] Sellers, R. F., & Taylor, W. P. (1980). Epidemiology of bluetongue and the import and export of livestock, semen and embryos [sheep, virus, domestic ruminants, *Culicoides*]. *Bulletin de l'Office International des Epizooties*. *92*(1), 587-592.
[65] Ribeiro, R., Wilson, A.J., Nunes, T., Ramilo, D.W., Amador, R., Madeira, S., Baptista, F.M., Lucientes, J., & Boinas, F. (2015). Spatial and temporal distribution of *Culicoides* species in mainland Portugal (2005-2010). Results of the Portuguese Entomological Surveillance Program. *PLoS One*, *10*(4), e0124019.
[66] Barros, S. C., Ramos, F., Luís, T. M., Vaz, A., Duarte, M., Henriques, M., Cruz, B., & Fevereiro, M. (2007). Molecular epidemiology of bluetongue virus in Portugal during 2004–2006 outbreak. *Veterinary microbiology*, *124*(1-2), 25-34.
[67] DGAV | Direção Geral de Alimentação e Veterinária (2019). *Língua Azul – Febre Catarral Ovina*. Accessed on the 2nd December, 2019, available in: http://www.dgv.min-agricultura.pt/portal/page/portal/DGV/
[68] Crespo, A. P. M. A. M. (2012). Controlo de pragas no Jardim Zoológico de Lisboa: particular relevância para o controlo de roedores e sua infeção parasitária. *Master's dissertation in Food Security. Universidade de Lisboa, Faculdade de Medicina Veterinária, Lisboa.*
[69] Visit Portugal (2019). Destinos. Accessed on the 12th June, 2019, available in: https://www.visitportugal.com/pt-pt/destinos
[70] Jardim Zoológico de Lisboa (2019). *Mapa do ZOO*. Accessed on the 12th June, 2019, available in: https://www.zoo.pt/pt/visitar/mapa-do-zoo/
[71] Centro Hospitalar de Leiria (2019). *Área de Influência – Mapa de Leiria*. Accessed on the 12th June, 2019, available in: http://www.chleiria.pt/galeria/images/mapa_area_influencia(2).png
[72] Wikipedia (2019). *Mapa de Lisboa – Ajuda*. Accessed on the 12th June, 2019, available in: https://pt.wikipedia.org/wiki/Ajuda_(Lisboa)
[73] Ramilo, D. Wpp., Diaz, S., Pereira da Fonseca, I., Delécolle, J.C., Wilson, A., Meireles, J., Lucientes, J., Ribeiro, R., & Boinas, F. (2012) First Report of 13 Species of *Culicoides* (Diptera: Ceratopogonidae) in Mainland Portugal and Azores by Morphological and Molecular Characterization. *PLoS One 7(4),* e34896.
[74] Mignotte, A., Garros, C., Gardès, L., Balenghien, T., Duhayon, M., Rakotoarivony, I., Tabourin, L., Poujol, L., Mathieu, B., Ibañez-Justicia, A., & Deniz, A. (2020). The tree that hides the forest: cryptic diversity and phylogenetic relationships in the Palaearctic vector Obsoletus/Scoticus Complex (Diptera: Ceratopogonidae) at the European level. *Parasites & vectors*, *13*(1), 1-13.
[75] Mullis, K. B. (1990). The unusual origin of the polymerase chain reaction. *Scientific American*, *262*(4), 56-65.
[76] Garibyan, L., & Avashia, N. (2013). Polymerase chain reaction. *The Journal of investigative dermatology*, *133*(3), 1–4.
[77] Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., & Flook, P. (1994). Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the entomological Society of America*, *87*(6), 651-701.
[78] Lee, P. Y., Costumbrado, J., Hsu, C. Y., & Kim, Y. H. (2012). Agarose gel electrophoresis for the separation of DNA fragments. *Journal of visualized experiments: JoVE*, (62), 3923.
[79] Voytas, D. (2000). Agarose gel electrophoresis. *Current protocols in molecular biology*, *51*(1), 2-5.

[80] Smith, D. R. (1996). Agarose gel electrophoresis. *Basic DNA and RNA Protocols: Methods in Molecular Biology*, (58), 17-21. Totowa: Humana Press Inc.
[81] McHugh, M. L. (2013). The chi-square test of independence. *Biochemia medica: Biochemia medica*, *23*(2), 143-149.
[82] Silva, F. S., & Carvalho, L. P. C. (2013). A population study of the *Culicoides* biting midges (Diptera: Ceratopogonidae) in urban, rural, and forested sites in a cerrado area of Northeastern Brazil. *Annals of the Entomological Society of America*, *106*(4), 463-470.
[83] Pena, I. (2004). Contribuição para o estudo da sistemática, biologia e ecologia dos *Culicoides* (Diptera: Ceratopogonidae) de Portugal: Sua importância médica e veterinária. *Ph.D. Thesis in Biology: Ecology and Sistematics. Universidade da Madeira. Faculdade de Ciências da Vida, Funchal.*
[84] Talavera, S., Muñoz-Muñoz, F., Durán, M., Verdún, M., Soler-Membrives, A., Oleaga, Á., Arenas, A., Ruiz-Fons, F., Estrada, R., & Pàges, N. (2015). *Culicoides* species communities associated with wild ruminant ecosystems in Spain: tracking the way to determine potential bridge vectors for arboviruses. *PLoS One*, *10*(10).
[85] Rádrová, J., Mračková, M., Galková, Z., Lamka, J., Račka, K., Barták, P., & Votýpka, J. (2016). Seasonal dynamics, parity rate, and composition of *Culicoides* (Diptera: Ceratopogonidae) occurring in the vicinity of wild and domestic ruminants in the Czech Republic. *Journal of medical entomology*, *53*(2), 416-424.
[86] De Regge, N., De Deken, R., Fassotte, C., Losson, B., Deblauwe, I., Madder, M., Vantieghem, P., Tomme, M., Smeets, F., & Cay, A. B. (2015). *Culicoides* monitoring in Belgium in 2011: analysis of spatiotemporal abundance, species diversity and Schmallenberg virus detection. *Medical and veterinary entomology*, *29*(3), 263-275.
[87] Calvo, J. H., Berzal, B., Calvete, C., Miranda, M. A., Estrada, R., & Lucientes, J. (2012). Host feeding patterns of *Culicoides* species (Diptera: Ceratopogonidae) within the Picos de Europa National Park in northern Spain. *Bulletin of entomological research*, *102*(6), 692-697.
[88] Santiago-Alarcon, D., Havelka, P., Schaefer, H. M., & Segelbacher, G. (2012). Bloodmeal analysis reveals avian Plasmodium infections and broad host preferences of *Culicoides* (Diptera: Ceratopogonidae) vectors. *PLoS One*, *7*(2).
[89] Ortega, M. D., Holbrook, F. R., & Lloyd, J. E. (1999). Seasonal distribution and relationship to temperature and precipitation of the most abundant species of *Culicoides* in five provinces of Andalusia, Spain. *Journal of the American Mosquito Control Association-Mosquito News*, *15*(3), 391-399.
[90] Mellor, P. S., & Boorman, J. (1995). The transmission and geographical spread of African horse sickness and bluetongue viruses. *Annals of Tropical Medicine & Parasitology*, *89*(1), 1-15.
[91] England, M. E., Pearce-Kelly, P., Brugman, V. A., King, S., Gubbins, S., Sach, F., Sanders, C.J., Masters, N.J., Denison, E., & Carpenter, S. (2020). *Culicoides* species composition and molecular identification of host blood meals at two zoos in the UK. *Parasites & Vectors*, *13*(1), 1-13.
[92] Labuschagne, K., Gerber, L. J., Espie, I., & Carpenter, S. (2007). *Culicoides* biting midges at the National Zoological Gardens of South Africa: research communication. *Onderstepoort Journal of Veterinary Research*, *74*(4), 343-347.
[93] Vilar, M. J., Guis, H., Krzywinski, J., Sanderson, S., & Baylis, M. (2011). *Culicoides* vectors of bluetongue virus in Chester Zoo. *Veterinary Record*, *168*(1), 242.
[94] Bobeva, A., Zehtindjiev, P., Ilieva, M., Dimitrov, D., Mathis, A., & Bensch, S. (2015). Host preferences of ornithophilic biting midges of the genus *Culicoides* in the Eastern Balkans. *Medical and veterinary entomology*, *29*(3), 290-296.
[95] Bartsch, S., Bauer, B., Wiemann, A., Clausen, P. H., & Steuber, S. (2009). Feeding patterns of biting midges of the *Culicoides obsoletus* and *Culicoides pulicaris* groups on selected farms in Brandenburg, Germany. *Parasitology research*, *105*(2), 373-380.
[96] Martínez-de la Puente, J., Figuerola, J., & Soriguer, R. (2015). Fur or feather? Feeding preferences of species of *Culicoides* biting midges in Europe. *Trends in parasitology*, *31*(1), 16-22.
[97] Yavru, S., Dik, B., Bulut, O., Uslu, U., Yapici, O., Kale, M., & Avci, O. (2018). New *Culicoides* vector species for BTV transmission in Central and Central west of Anatolia. *Annual Research & Review in Biology*, *27*(1), 1-9.

[98] Foxi, C., Pinna, M., Monteys, V. S. I., & Delrio, G. (2011). An updated checklist of the *Culicoides* Latreille (Diptera: Ceratopogonidae) of Sardinia (Italy), and seasonality in proven and potential vectors for bluetongue virus (BTV). *Proceedings of the Entomological Society of Washington*, *113*(4), 403-416.
[99] Barceló, C., Estrada, R., Lucientes, J., & Miranda, M. A. (2020). A Mondrian matrix of seasonal patterns of *Culicoides* nulliparous and parous females at different latitudes in Spain. *Research in Veterinary Science*, *129*(1), 154-163.
[100] IPMA | Instituto Português do Mar e da Atmosfera (2020). IPMA – Boletim Climatológico Anual – Ano 2010. Updated on the 4th February 2011. Accessed on the 25th March 2020, available in: https://www.ipma.pt/resources.www/docs/im.publicacoes/edicoes.online/20110204/PdTzSQuJAvrrwvtcdtee/cli_20100101_20101231_pcl_aa_co_pt.pdf
[101] WMO | World Meteorological Organization (2020). WMO – Press Release. Updated on the 15th January 2020. Accessed on the 25th March 2020, available in: https://public.wmo.int/en/media/press-release/wmo-confirms-2019-second-hottest-year-record
[102] IPMA | Instituto Português do Mar e da Atmosfera (2020). IPMA – Resumo Climático – Ano 2019. Updated on the 24th January 2020. Accessed on the 25th March 2020, available in: http://www.ipma.pt/resources.www/docs/im.publicacoes/edicoes.online/20200124/duxzjqIIDrBxNidAOAXo/cli_20191201_20191231_pcl_aa_co_pt.pdf
[103] Guichard, S., Guis, H., Tran, A., Garros, C., Balenghien, T., & Kriticos, D. J. (2014). Worldwide niche and future potential distribution of *Culicoides imicola*, a major vector of bluetongue and African horse sickness viruses. *PLoS One*, *9*(11), e112491.
[104] Savini, G., Goffredo, M., Monaco, F., Di Gennaro, A., Cafiero, M. A., Baldi, L., P. De Santis, R. Meiswinkel & Caporale, V. (2005). Bluetongue virus isolations from midges belonging to the Obsoletus complex (*Culicoides*, Diptera: Ceratopogonidae) in Italy. *Veterinary Record*, *157*(5), 133-139.
[105] Kjær, L. J., Soleng, A., Edgar, K. S., Lindstedt, H., Paulsen, K. M., Andreassen, Å. K., Korslund, L., Kjelland, V., Slettan, A., Stuen, S., Kjellander, P., Christensson, M., Teräväinen, M., Baum, A., Klitgaard, K., & Bødker, R. (2019). Predicting and mapping human risk of exposure to *Ixodes ricinus* nymphs using climatic and environmental data, Denmark, Norway and Sweden, 2016. *Euro surveillance: bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin*, *24*(9), 1800101.
[106] Conte, A., Goffredo, M., Ippoliti, C., & Meiswinkel, R. (2007). Influence of biotic and abiotic factors on the distribution and abundance of *Culicoides imicola* and the Obsoletus Complex in Italy. *Veterinary parasitology*, *150*(4), 333-344.
[107] Nielsen, S. A., Nielsen, B. O., & Chirico, J. (2010). Monitoring of biting midges (Diptera: Ceratopogonidae: *Culicoides* Latreille) on farms in Sweden during the emergence of the 2008 epidemic of bluetongue. *Parasitology research*, *106*(5), 1197-1203.
[108] Möhlmann, T. W. R., Bekendam, A. M., van Kemenade, I., Wennergren, U., Favia, G., Takken, W., & Koenraadt, C. J. M. (2019). Latitudinal diversity of biting midge species within the Obsoletus group across three habitats in Europe. *Medical and veterinary entomology*, *33*(3), 420-426.
[109] Smith, W. W., & Perry, V. G. (1967). Intersexes in *Culicoides* spp. caused by mermithid parasitism in Florida. *Journal of Economic Entomology*, *60*(4), 1025-1027.
[110] Felippe-Bauer, M. L., & Silva, C. S. (2006). Morphological alterations in Neotropical Ceratopogonidae (Diptera). *Revista Brasileira de Zoologia*, *23*(3), 593-596.
[111] Lorenz, T. C. (2012). Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. *JoVE (Journal of Visualized Experiments)*, (63), e3998.

8. ANNEXES

8.1. Captures data

8.1.1. Sylvatic Animals from Lisbon Zoo (LZ)

Annex 1a – *Culicoides* species captured near sylvatic animals (giraffes, zebras and birds).

***Culicoides* Species**	**Female**
C. parroti	22
C. circumscriptus	9
C. punctatus	25
C. imicola	833
C. gejgelensis	24
C. santonicus Callot, Kremer, Rault & Bach, 1966	3
C. puncticollis	3
C. nubeculosus	29
C. univittatus	2
C. bahrainensis Boorman, 1989	5
C. newsteadi	9
C. odiatus Austen, 1921	1
C. pseudopallidus Khalaf, 1961	1
C. haranti Rioux, Descous & Pech, 1959	1
C. begueti Clastrier, 1957	1
C. obsoletus	106✯
C. scoticus	14

✯ – 2 of those 106 specimens were found with morphological anomalies.

Annex 1b – *Culicoides* species captured near giraffes, by month.

Giraffes																		
Female *Culicoides* Species	**Month (2018-2019)**																	
	May 18	**June 18**	**July 18**	**August 18**	**September 18**	**October 18**	**November 18**	**December 18**	**January 19**	**February 19**	**March 19**	**April 19**	**May 19**	**June 19**	**July 18**	**August 18**	**September 18**	**Total**
C. parroti	3	1	0	1	0	0	0	0	0	0	0	0	1	0	3	0	13	22
C. circumscriptus	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8
C. punctatus	11	1	0	0	0	0	0	0	2	0	3	0	3	0	2	0	1	24
C. imicola	12	42	7	310	81	28	11	0	0	0	2	0	0	1	8	0	86	587
C. gejgelensis	6	10	0	2	0	0	0	0	0	0	0	0	0	0	0	0	4	22
C. santonicus	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
C. puncticollis	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
C. nubeculosus	5	2	1	9	2	0	0	0	0	0	0	0	0	0	0	0	10	29
C. univittatus	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
C. bahrainensis	1	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	5
C. newsteadi	0	4	0	3	0	0	0	0	0	0	0	0	0	1	0	0	1	9
C. odiatus	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
C. pseudopallidus	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
C. scoticus	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
C. obsoletus	11	12	0	0	3	1	1	0	0	0	0	1	1	0	1	1	3	46[☆]
C. haranti	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1

[☆] – 1 of those 46 specimens was found with morphological anomalies.

Annex 1c – *Culicoides* species captured near zebras, by month.

Zebras																		
Female *Culicoides* Species	**Month (2018-2019)**																	
	May 18	**June 18**	**July 18**	**August 18**	**September 18**	**October 18**	**November 18**	**December 18**	**January 19**	**February 19**	**March 19**	**April 19**	**May 19**	**June 19**	**July 18**	**August 18**	**September 18**	**Total**
C. imicola	4	0	6	0	110	125	0	0	0	0	0	0	0	0	0	0	0	245
C. scoticus	0	0	5	0	1	2	0	0	0	0	0	0	0	0	0	0	0	9
C. obsoletus	6	1	20	0	9	12	0	0	0	1	0	0	1	0	1	1	0	52[☆]
C. begueti	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1

[☆] – 1 of those 52 specimens was found with morphological anomalies.

Annex 1d – *Culicoides* species captured near birds, by month.

Birds																		
Female *Culicoides* Species	**Month (2018-2019)**																	
	May 18	**June 18**	**July 18**	**August 18**	**September 18**	**October 18**	**November 18**	**December 18**	**January 19**	**February 19**	**March 19**	**April 19**	**May 19**	**June 19**	**July 18**	**August 18**	**September 18**	**Total**
C. circumscriptus	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
C. punctatus	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
C. imicola	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
C. gejgelensis	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	2
C. obsoletus	0	0	4	0	0	0	0	0	0	0	2	0	2	0	0	0	0	8
C. scoticus	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1

8.1.2. Domestic Cattle from Leiria and Lisbon

Annex 2a – *Culicoides* species captured near domestic cattle in Leiria District, by month.

Female *Culicoides* Species	**Month (2010)**			**Total**
	June	**July**	**September**	
C. acharyi	1	0	0	1
C. heteroclitus	0	0	1	1
C. indistinctus	0	1	0	1
C. newsteadi	46	9	1	56
Grupo Obsoletus	1755	622	1	2378
C. pulicaris	167	276	2	445
C. punctatus	326	134	2	462
C. fascipennis	2	0	1	3

Annex 2b – Obsoletus group species used for this project near domestic cattle in Leiria District to search for morphological anomalies.

Female *Culicoides* Species	**Month (2010)**			
	February	**April**	**July**	**Total**
Obsoletus group	72	6730	622	7424
C. obsoletus	2	4	30	36✯✯
C. scoticus	13	11	46	70✯

106 species from Obsoletus group: 70 *C. scoticus* and 36 *C. obsoletus* (including morphological anomalies).

✯ – 2 of those 70 species were found with morphological anomalies.

✯✯ – 1 of those 36 species was found with morphological anomalies.

Annex 2c – *Culicoides* species captured near domestic cattle in Lisbon (FMV-ULisbon), by month.

Female *Culicoides* Species	**Month (2019)**			**Total**
	June	**July**	**September**	
C. newsteadi	2	0	0	2
C. scoticus	37	3	3	43
C. obsoletus	70	0	15	85✯
C. pulicaris	1	0	0	1
C. punctatus	15	1	5	21
C. paolae	0	0	1	1
C. imicola	2	0	3	5

✯ – 8 of those 85 specimens were found with morphological anomalies.

8.2. *Rstudio* Script

8.2.1. Sylvatic animals vs. Domestic ruminants

```
dados = read.csv("C:/Users/User/Desktop/scripts Rstudio/sylvsdom.csv", header=T, row.names=1, sep=";")
boxplot(Number.of.Culicoides.Species ~ Local, data=dados, col=
        colors(distinct = FALSE),main="Sylvatic animals vs Domestic ruminants", xlab= "Local",
       ylab="Captured Culicoides Specimens", ylim=c(0,95), xlim=c(0,3))
LZ=dados[c(1:8),]
summary(LZ)
LZ$Number.of.Culicoides.Species
DOM=dados[c(9:15),]
summary(DOM)
DOM$Number.of.Culicoides.Species
dadostable = xtabs(Number.of.Culicoides.Species ~ Culicoides.Species+Local, data=dados)
summary(dadostable)
View(dadostable)
fisher.test(x=dadostable, simulate.p.value = T)
```

8.2.2. Sylvatic animals: birds vs. giraffes vs. zebras

```
dados = read.csv("C:/Users/User/Desktop/scripts Rstudio/excel_total.csv", header=T, row.names=1, sep=";")
zoo = dados[dados[,"Local"] == "LZ",]
zoo = droplevels(zoo)
boxplot(Number.of.Culicoides.Species ~ Animal.Species, data=zoo, col= colors(distinct = FALSE),main="Sylvatic Animals: birds vs giraffes vs zebras", ylab=" Captured Culicoides Specimens ", ylim=c(0,600), xlim=c(0,4))
giraffes=zoo[c(1:16),]
summary(giraffes)
giraffes$Number.of.Culicoides.Species
zebras=zoo[c(17:20),]
```

```
summary(zebras)
zebras$Number.of.Culicoides.Species
birds=zoo[c(21:26),]
summary(birds)
birds$Number.of.Culicoides.Species
zootable = xtabs(Number.of.Culicoides.Species ~ Culicoides.Species+Animal.Species, data=zoo)
summary(zootable)
View(zootable)
fisher.test(x=zootable, simulate.p.value = T)
```

8.2.3. Domestic cattle: Leiria vs. Faculty of Veterinary Medicine (FMV-ULisbon)

```
dados = read.csv("C:/Users/User/Desktop/scripts Rstudio/DOMoldvsrecent.csv", header=T,
row.names=1, sep=";")
cows = dados[dados[,"Animal.Species"] == "Cows",]
cows = droplevels(cows)
boxplot(Number.of.Culicoides.Species ~ Local, data=cows, col= colors(distinct =
FALSE),main="Domestic Ruminants: Old Captures vs Recent Captures", ylab="Captured Culicoides
Specimens", xlab="Local", ylim=c(0,2380), xlim=c(0,3))
LEIRIA=cows[(1:8),]
summary(LEIRIA)
LEIRIA$Number.of.Culicoides.Species
FMV=cows[(9:14),]
summary(FMV)
FMV$Number.of.Culicoides.Species
cowstable= xtabs(Number.of.Culicoides.Species ~ Culicoides.Species+Local, data = cows)
summary(cowstable)
View(cowstable)
fisher.test(x=cowstable, simulate.p.value = T)
```